Cardiac contractile actions of prostaglandin F2alpha

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Cardiac Contractile Actions of Prostaglandin $F_{2\alpha}$

Submitted by Yew Su Fong

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Adrian Lee kept me sane. Nick Edmunds, Kate Reeves, Harbans Lal and many others shared laughter and friendship.

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For my parents

Summary

This study was done to characterise the mechanism of action for the positive inotropic effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in rat hearts. In Langendorff non-working hearts perfused at a constant flow rate, $PGF_{2\alpha}$ increased cardiac contractility in a dose dependent manner. The $PGF_{2\alpha}$ analogue selective for FP receptors, cloprostenol, had a similar effect on cardiac contractility. Both drugs dilated coronary vessels, and with higher doses of $PGF_{2\alpha}$, coronary constriction preceded the dilator response. Heart rate was unchanged. Contractility of paced left atrial preparations also increased with $PGF_{2\alpha}$.

Single cell experiments were carried out to determine whether the mechanism of action of the $PGF_{2\alpha}$-induced positive inotropy depended on changes in intracellular Ca^{2+}. Myocyte shortening was monitored by edge detection, while Ca^{2+} transients and intracellular pH were monitored with the fluorescent probes, fura-2 and BCECF, respectively. At 3 μM, $PGF_{2\alpha}$ increased the shortening amplitude of fura-2 loaded ventricular myocytes by 40%, but it did not increase the amplitude of intracellular Ca^{2+} transients. While diastolic cell length decreased with the same concentration of $PGF_{2\alpha}$, diastolic Ca^{2+} did not change. These two factors suggest that the sensitivity of the contractile filaments to Ca^{2+} were enhanced. Neither the duration of the Ca^{2+} transients, nor that of the contractions was affected.

However, a significant rise in intracellular pH of 0.08 pH units was detected in BCECF loaded myocytes with 3 μM $PGF_{2\alpha}$. This alkalosis was abolished in the presence of HOE 694, a Na^+-H^+ exchange inhibitor, and attenuated by the protein kinase C (PKC) inhibitor, chelerythrine. Parallel experiments showed that the $PGF_{2\alpha}$-induced increase in myocyte shortening was also abolished by HOE 694, and attenuated by chelerythrine. Hence, the positive inotropic effect of $PGF_{2\alpha}$ appears to be mediated by activation of PKC and the Na^+-H^+ exchanger, resulting in an intracellular alkalosis that sensitises the contractile filaments to Ca^{2+}.

Abbreviations

ANF	atrial natriuretic factor
BCECF/AM	acetoxymethyl ester derivative of 2',7'-bis(carboxyethyl)-5,6-carboxyfluorescein
cAMP	cyclic 5'adenosine monophosphate
CLO	cloprostenol
DMSO	dimethylsulphoxide
ERK	extracellular signal-regulated kinase
FPRP	prostaglandin $F_{2\alpha}$ receptor associated protein
HEPES	(N-[2-hydroxyethyl]piperazine-N'-{2-ethanesulphonic acid})
IP_3	inositol 2,4,5-trisphosphate
MAPK	mitogen activated protein kinase
MLCK	myosin light chain kinase
NAd	noradrenaline
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PIP	phosphatidylinositol 4-phosphate
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKC	protein kinase C
PLC	phospholipase C
PLD	phospholipase D
SNARF	seminaphthorhodafluor

Contents

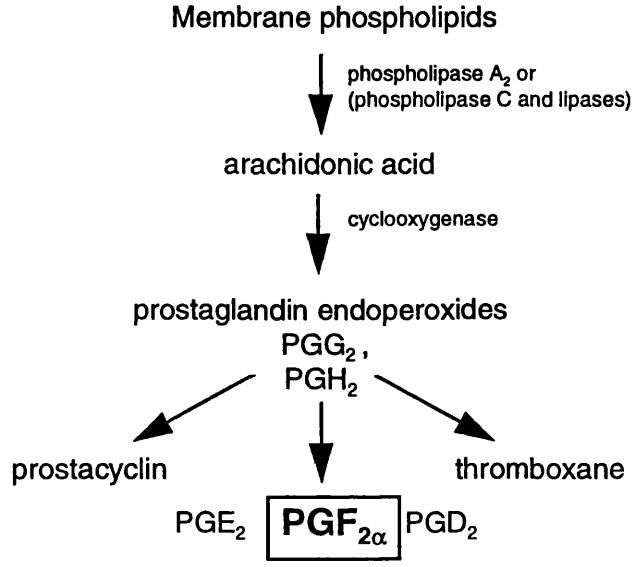
SECTION 1

Introduction

1.1 Prostaglandin $F_{2\alpha}$ and the heart

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) is one of the many endogenous modulators of cardiac contractility. It belongs to the prostanoid family of fatty acids, derived by cyclooxygenase mediated breakdown of arachidonic acid (diagram I-1). Bergstrom & Sjovall first isolated $PGF_{2\alpha}$ from sheep prostate glands in 1957. Since then, it has been found in many tissues, including those of non-mammalian vertebrates, invertebrates, fish and insects (Moore, 1985). In the heart, formation of $PGF_{2\alpha}$ occurs mainly in coronary vessels (Sivakoff *et al.*, 1979), but cardiac myocytes also synthesise $PGF_{2\alpha}$ (Ahumada *et al.*, 1980).

Diagram I-1. Pathway of $PGF_{2\alpha}$ synthesis



$PGF_{2\alpha}$ is the most potent prostaglandin in increasing cardiac contractility (Vergroeson *et al.*, 1967; Metsa-Ketela, 1981). This action of $PGF_{2\alpha}$ may be important under conditions of ischaemia where contractility is compromised, as the release of this prostaglandin is significantly increased. Human hearts made temporarily ischaemic by rapid pacing are associated with almost a three-fold increase of $PGF_{2\alpha}$ in the coronary sinus blood (Berger *et al.*, 1977), whereas rat hearts with infarction and hypertrophy have six-fold more extractable $PGF_{2\alpha}$ than control hearts (Lai *et al.*, 1996).

Besides affecting cardiac contractility, $PGF_{2\alpha}$ also influences other cardiac functions. It constricts porcine and canine coronary vessels (Balwierczak, 1991; Rigel & Shetty, 1997), and modulates cardiac rhythm. In rat hearts, low concentrations (pM-nM) of $PGF_{2\alpha}$ are reported to be arrhythmic, while higher concentrations normalise the rhythm (Swift *et al.*, 1978). Others have also shown that $PGF_{2\alpha}$ modulates chemically-induced arrhythmias (Forster *et al.*, 1973; Vapaatalo *et al.*, 1978; Moffat *et al.*, 1987). In a preliminary clinical trial, intravenous infusion of $PGF_{2\alpha}$ abolished or reduced extrasystoles in five out of six patients (Mann *et al.*, 1973). $PGF_{2\alpha}$ also releases atrial natriuretic factor, ANF, from rat hearts and ventricular myocytes (Rayner *et al.*, 1993; Lai *et al.*, 1996), and causes ventricular hypertrophy (Adams *et al.*, 1996; Lai *et al.*, 1996). In fact, $PGF_{2\alpha}$ produces about ten times more ANF than other hypertrophic agents such as phenylephrine and endothelin-1 (Lai *et al.*, 1996). Additionally, $PGF_{2\alpha}$ contributes to reperfusion injury of ischaemic rat hearts (Karmazyn, 1986).

In view of $PGF_{2\alpha}$'s complex cardiac actions and its enhanced release during myocardial ischaemia, the primary aim of this study is to determine the mechanism mediating the $PGF_{2\alpha}$-induced increase in cardiac contractility. If this could be elucidated, then it would hopefully add to our understanding of how cardiac contractility is normally controlled, and how this mechanism could contribute towards $PGF_{2\alpha}$'s other cardiac actions.

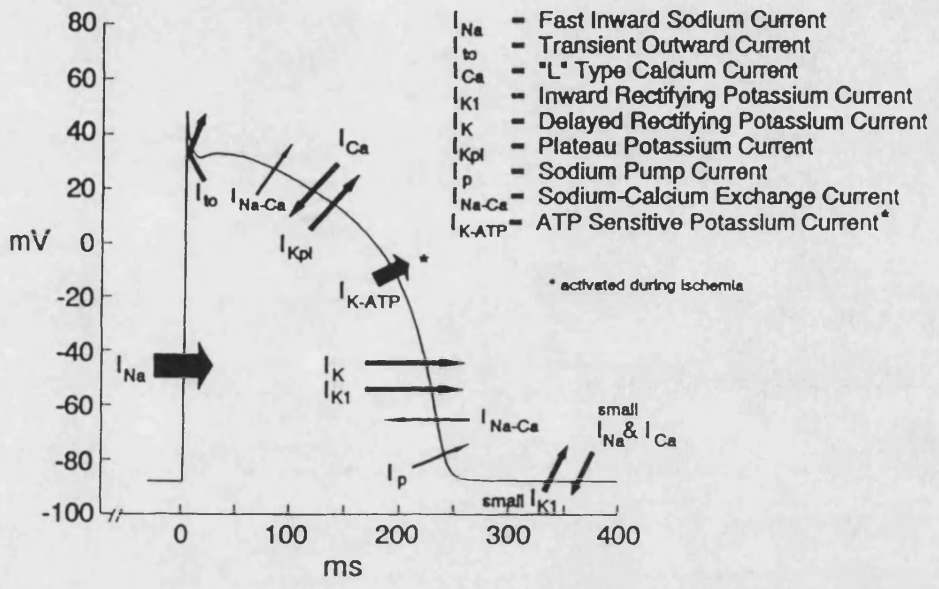
An increase in cardiac contractility in the absence of preload and afterload is termed 'positive inotropic'. It is defined as 'an increase in work performed during the cardiac cycle not caused by a change in initial fibre length' (Katz, 1992). The positive inotropic action of $PGF_{2\alpha}$ may be achieved by either an increase in intracellular Ca^{2+} or an increase in the sensitivity of the contractile filaments to Ca^{2+}. The basis underlying cardiac contraction and the possible mechanisms of the positive inotropic action of $PGF_{2\alpha}$ are considered in the following sections.

1.2 Cardiac action potentials

Cardiac contractility is dependent on action potentials that are generated by electrical activity normally derived from the sinus node, and propagated along conductive tissues. The sarcolemmal membrane potential is normally maintained by the Na^+/K^+ ATPase and the inward rectifier K^+ current at around -80 mV. During an action potential (reviewed by Ten Eick *et al.*, 1992), depolarisation beyond about -70 mV activates a large, rapidly inactivating Na^+ current (I_{Na}) that manifests in the upstroke of the action potential (diagram I-2). This Na^+ influx depolarises the cells and activates voltage dependent L-type Ca^{2+} channels. Following the peak of the upstroke, rapid repolarisation occurs primarily from inactivation of this Na^+ current and activation of a transient outward current carried by K^+ and Cl^- (I_{to}). The subsequent plateau phase arises from a balance between the inward L-type Ca^{2+} current (I_{Ca}) and plateau-activated outward K^+ current (I_{Kpl}). Ca^{2+} entry through Na^+-Ca^{2+} exchange ($I_{Na\text{-}Ca}$) may also occur. It is the entry of Ca^{2+} occurring during the action potential plateau that activates cardiac contraction. Inactivation of the Ca^{2+} current and repolarisation by outward K^+ currents (delayed rectifier I_K, and inwardly rectified I_{K1}) then return membrane potential to the resting level.

Action potentials vary in their shape depending on species and on their location in the myocardium. For example, the transient outward K^+ current is more pronounced in atrial than in ventricular myocytes. Hence, the plateau of the action potential is much less pronounced in atrial myocytes (Berne & Levy, 1992).

Diagram I-2. An action potential recorded from an isolated feline ventricular myocyte using the patch electrode. Inward currents are depicted by arrows directed "into the action potential" and outward currents directed by arrows "out of the action potential". Taken from Ten Eick *et al.* (1992).



1.3 The importance of Ca^{2+} in cardiac contractility

Contraction of a single cardiac myocyte is dependent on the force generated between the cytosolic thick filamentous protein, myosin, and thin filamentous protein, actin, during the 'cross-bridge' cycle (see diagram I-3 below). Before this cycle begins, the ATP-bound myosin is unattached to actin. Interaction between actin and myosin is hindered by the troponin complex and tropomyosin, both of which also make up the thin filaments. Troponin T attaches the troponin complex to actin and tropomyosin, troponin I inhibits the actin-myosin interaction, while troponin C binds Ca^{2+}.

It is the binding of Ca^{2+} to troponin C in the presence of ATP and Mg^{2+} that triggers the cross-bridge cycle. Conformation of the troponin complex then changes so that tropomyosin shifts out of the way and interaction of actin to myosin occurs. The globular heads of myosin attach actin, flex, and hydrolyse ATP. Repetition of this cross-bridge cycle at many points along each filament (myofibrillar ATPase activity) produces the force that causes sliding of the filaments past one another, which results in myocyte contraction. Relaxation follows when Ca^{2+} leaves troponin C, and the myosin heads release actin.

Diagram I-3. Diagram depicting the ultrastructure of thin and thick myofilaments of cardiac muscle. Bold P indicates a site of covalent phosphorylation. The proteins that make up the troponin complex are troponin T, the tropomyosin binding unit; troponin I, the inhibitory unit; and troponin C, the Ca^{2+} binding unit. Taken from Solaro, 1993.

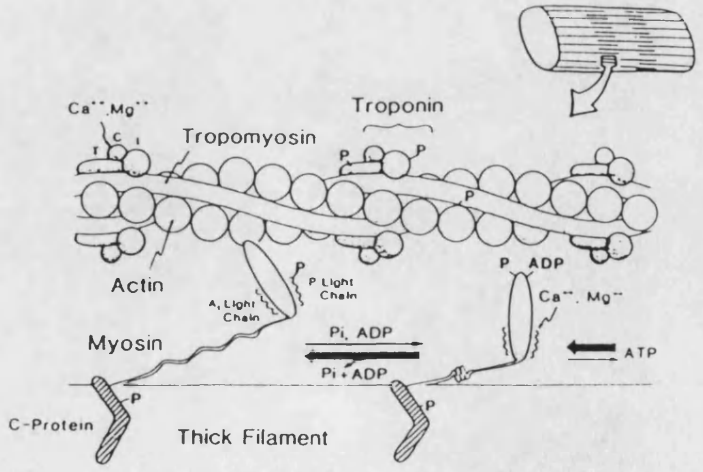
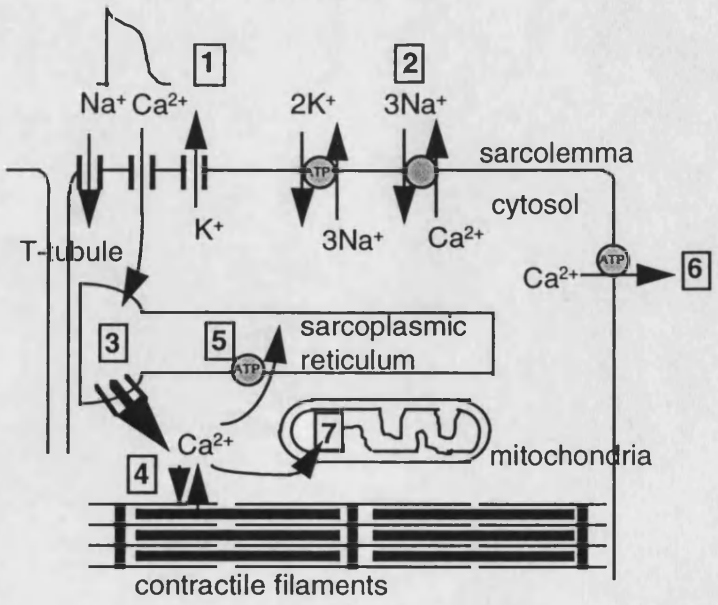


Diagram I-4 (1-7) shows the main factors involved in regulating cell Ca^{2+}. During an action potential (1), Ca^{2+} influx occurs through L-type Ca^{2+} channels and perhaps the Na^{+}/Ca^{2+} exchanger (2). This amount of Ca^{2+} is generally insufficient to activate the contractile proteins in mammalian cardiac muscle, but it triggers more Ca^{2+} release from the sarcoplasmic reticulum via the ryanodine receptors, a process known as 'Ca^{2+}-induced Ca^{2+} release' (3). This cytosolic Ca^{2+} then binds to intracellular sites that include troponin C, calmodulin and the sarcoplasmic reticulum. Contraction is initiated when Ca^{2+} binds to troponin C (4). Inactivation of the Ca^{2+} current and repolarisation by outward K^{+} currents (delayed rectifier, and inwardly rectified K^{+} currents) inhibit further Ca^{2+} entry. For relaxation to occur, Ca^{2+} leaves troponin C, and the free cytosolic Ca^{2+} concentration is then reduced by several mechanisms. Active sarcoplasmic reticulum Ca^{2+} uptake through the Ca^{2+} ATPase replenishes sarcoplasmic reticulum Ca^{2+} stores (5). Ca^{2+} is also extruded through both the sarcolemmal Na^{+}-Ca^{2+} exchanger and the Ca^{2+} ATPase (6), and when Ca^{2+} overload occurs, the mitochondria also becomes a sink for Ca^{2+} (7).

Diagram I-4. Ion fluxes in cardiac muscle. See text above for details. Note that K^{+} efflux occurs through several types of K^{+} channels.



1.4 Regulation of cardiac contractility at the cellular level

Manipulations that either increase cytosolic Ca^{2+} or increase Ca^{2+} sensitivity of the contractile proteins will increase contractility. These processes are either modulated directly at the sites discussed below or indirectly at the receptor and second messenger level.

a) Processes increasing cytosolic Ca^{2+}

An increased Ca^{2+} level can be achieved by several ways. Firstly, increased L-type Ca^{2+} channel activity will increase Ca^{2+} entry into the cell. Also, reduced K^+ efflux during an action potential will decrease the membrane potential and prolong Ca^{2+} influx. Secondly, increased Ca^{2+} influx, or reduced Ca^{2+} efflux can occur by manipulation of the Na^+/Ca^{2+} exchanger. Reduction of the Na^+ gradient across the cell membrane occurring during Na^+/K^+ ATPase inhibition, and possibly Na^+ channel or Na^+/H^+ exchanger activation, will also affect Na^+/Ca^{2+} exchanger activity. Reduced Ca^{2+} extrusion by the sarcolemmal Ca^{2+} ATPase can also increase cytosolic Ca^{2+}. Thirdly, increasing sarcoplasmic reticulum Ca^{2+} uptake, therefore increasing Ca^{2+} loading and release, will increase cell Ca^{2+}. Phosphorylation of phospholamban, a protein that inhibits the sarcoplasmic reticulum Ca^{2+} ATPase, enhances Ca^{2+} loading. Inhibition of the phosphatase regulating phospholamban will also increase Ca^{2+} stores. Additionally, Ca^{2+} release from the sarcoplasmic reticulum can also be modulated at the site of the ryanodine receptor, and more controversially through the IP_3 receptor.

b) Processes increasing Ca^{2+} sensitivity of the myofilaments

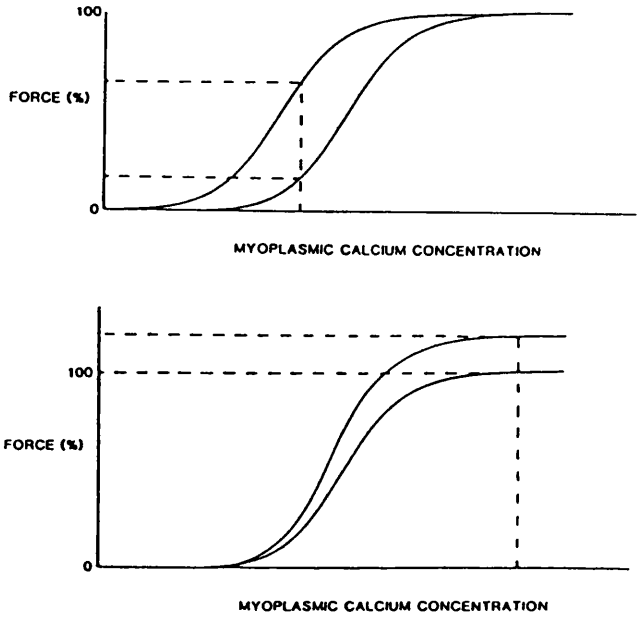
Ca^{2+} sensitivity of the contractile filaments can be regulated in the following ways. Firstly, the affinity of troponin C for Ca^{2+} can be enhanced, for example, by direct phosphorylation of troponin C, conformational changes of troponin C, or by cytosolic alkalosis. Secondly, Ca^{2+} sensitivity may be increased by altering myofibrillar ATPase activity, either by altering the number of cross-bridges or the force produced per cross-bridge. This will change the maximum Ca^{2+} activated force.

Both increased Ca^{2+} binding to troponin C and change in maximum Ca^{2+} activated force can be theoretically differentiated (Lee & Allen, 1993), as shown in diagram I-5 below.

In the top figure of diagram I-5, the right-hand curve shows the Ca^{2+}-force relation under control conditions, while the left-hand curve shows the effect of increasing the Ca^{2+} occupancy of troponin C at any given cell Ca^{2+}. This parallel leftward shift of the whole Ca^{2+}-force relation results in a greater generation of force at a given cell Ca^{2+} (illustrated by the dotted lines), and a lowered threshold Ca^{2+} for force generation. Relaxation of tension will therefore be slower under this condition because the off-rate of Ca^{2+} binding to troponin C is reduced.

The bottom figure shows the effects of changing maximum Ca^{2+} activated force. The Ca^{2+} force relation under control conditions is illustrated by the lower curve, while the upper curve shows the effects of increasing the maximum Ca^{2+} activated force. Not only is the maximum force increased, but the force response also saturates at the same cell Ca^{2+}. Additionally, the threshold for cell Ca^{2+} for force generation does not change.

Diagram I-5. Schematic representations of the effects of a pure change in Ca^{2+} sensitivity, for example, due to alteration of the Ca^{2+} affinity of troponin C (above) and a change in maximum Ca^{2+}-activated force (below). See text above for further discussion. Taken from Lee & Allen, 1993.



Change in Ca^{2+} affinity of troponin C

Change in maximum Ca^{2+} activated force

An example by which cardiac contractility can be modulated is by stimulation of the β_1-adrenergic receptor by isoprenaline or noradrenaline (Endoh & Blinks, 1988; Solaro, 1993). Activation of the β_1-adrenergic receptor results in cAMP-mediated protein kinase A stimulation. Protein kinase A phosphorylates L-type Ca^{2+} channels and phospholamban, and increases Ca^{2+} influx and sarcoplasmic reticulum Ca^{2+} loading respectively. This results in more Ca^{2+} being available for contraction. Additionally, protein kinase A phosphorylates troponin I and desensitises the contractile filaments to Ca^{2+}, and this, coupled with the enhanced Ca^{2+} loading of sarcoplasmic reticulum, shortens the duration of contraction.

Both the possibilities of increased cytosolic Ca^{2+} and increased Ca^{2+} sensitivity of the contractile proteins were considered in this thesis when studying the actions of $PGF_{2\alpha}$ in isolated perfused rat hearts, isolated atria and single ventricular myocytes.

1.5 The models and species used in this thesis

The actions of $PGF_{2\alpha}$ were first studied in isolated Langendorff perfused, non-working rat hearts. This model is not influenced by preload, afterload, circulating hormones or the peripheral nervous system. Besides contractility, several other parameters can also be measured in the whole heart, including heart rate, and coronary perfusion pressure, the latter of which gives a measure of coronary tone. However, the presence of fibrous tissue, the geometrical arrangement of muscle fibres and changes in coronary perfusion pressure ('garden hose effect') can affect cardiac contractility.

To overcome the influence of coronary perfusion pressure, the positive inotropic effect of $PGF_{2\alpha}$ was also studied in rat isolated atria and in single ventricular myocytes. The single myocyte model has an advantage over the atrial preparation as it is not influenced by substances released by other non-myocyte cells, or by hormones such as noradrenaline released from nerves during electrical stimulation. Additionally, single myocytes do not have diffusion barriers. Because of the technical difficulty in stretching intact myocytes, myocyte shortening, as opposed to force, is taken as a measure of contractility. Myocyte shortening can be recorded either by methods employing light diffraction techniques, or by direct monitoring of microscopic myocyte images (reviewed by Delbridge & Roos, 1997). In this thesis, the latter method was used.

The rat was used in the experiments in this thesis because besides being relatively cheap, previous studies have shown that it responds best to $PGF_{2\alpha}$ compared to other species. In fact, the positive inotropic action of $PGF_{2\alpha}$ was first observed by Vergroeson and his colleagues (1967) in the isolated perfused heart of this species. Subsequent experiments demonstrating this effect with $PGF_{2\alpha}$ in rat and guinea-pig hearts followed (see following pages, table I-1a,b). Dog, rabbit and human hearts, however, respond inconsistently to $PGF_{2\alpha}$ (table I-1c,d,h), while cat, chick and toad hearts do not show the positive inotropy (table I-1e,f,g). These species dependent responses may reflect the differences in abundance of the receptor or the signal transduction mechanisms involved in mediating the inotropic effect.

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

a) RAT

Authors	Preparation	$[PGF_{2\alpha}]$	Inotropic response?
Vergroeson *et al.*, 1967	isolated perfused heart	28 nM	positive
January & Schottelius, 1974	isolated left ventricular papillary muscle	28 nM & 280 nM	positive, increased rate of developed tension and rate of relaxation, decreased max. rate of membrane depolarisation, prolonged action potential duration
Vapaatalo *et al.*, 1978	isolated right atria	10 µM	positive, under conditions of either hypopotassemia or hyperpotassemia
Martinez *et al.*, 1977	cultured neonatal myocytes	0.1 µM & 10 µM	none, as determined by optical density changes
Metsa-Ketela, 1981	isolated right atria	50 µM	positive, $PGF_{2\alpha} > PGE_2 > PGE_1 \sim PGI$, biphasic, max. effect after 2.5 min, duration of contraction initially increased, no action on cAMP, atrial $^{45}Ca^{2+}$ accumulated
Karmazyn *et al.*, 1981	isolated perfused heart	28 pM – 280 nM	positive, sarcolemmal Na^+/K^+ ATPase, but not Ca^{2+} ATPase or Mg^{2+} ATPase, inhibited
Brenner *et al.*, 1987	myocyte cell culture	1 µM to 100 µM	negative
Otani *et al.*, 1988	isolated papillary muscle	1 nM – 1 µM (EC_{50} = 30 nM)	positive, phosphoinositide hydrolysis detected

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

b) GUINEA-PIG

Authors	Preparation	$[PGF_{2\alpha}]$ / Dose	Inotropic effect?
Sabatini – Smith, 1970	isolated left atria, fragmented sarcoplasmic reticulum	28 nM	positive, tissue Ca^{2+} unchanged but sarcoplasmic reticulum Ca^{2+} accumulation increased
Nutter & Ratts, 1973	isolated left atria, isolated right atria, right ventricular papillary muscles	28 µM 28 µM 42 – 84 µM	positive non-significant increase none
Forster *et al.*, 1974	isolated right atria, isolated papillary muscle	0.21 µM to 21 µM 2.1 µM to 21 µM	positive positive
Rettkowski *et al.*, 1978a	isolated right atria	28 µM	positive, in the presence of verapamil
Rettkowski *et al.*, 1978b	isolated atria	28 nM to 2.8 µM	increased cardiac work
Taube *et al.*, 1978	isolated perfused heart	10 µg bolus	negative, in parallel with reduced coronary flow
Mentz *et al.*, 1978	isolated atria	2.8 µM	positive, with a higher % increase in contractility at lower extracellular Ca^{2+}
Otani *et al.*, 1986	isolated right atria	0.1 – 10 µM	positive, unaffected by verapamil but inhibited by procaine or TMB-8. Unaffected by increased extracellular Ca^{2+}

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

c) DOG

Authors	Preparation	[$PGF_{2\alpha}$] / Dose	Inotropic response?
Ducharme *et al.*, 1968	anaesthetised animals	intravenous 10 µg/kg	none
Hollenberg *et al.*, 1968	anaesthetised animals	intracoronary 4 - 12 µg/min	none
Nakano, 1968	anaesthetised animals	intraarterial 0.1 µg/kg	none
Nakano & McCurdy, 1968	anaesthetised animals	intravenous 8 µg/kg	positive?
Emerson *et al.*, 1971	anaesthetised animals	inraarterial 58.2 µg/min (5 min)	positive?
Nutter & Crumly, 1972	anaesthetised animals	intracoronary 0.5 - 12.5 µg	positive
Bloor *et al.*, 1973	anaesthetised animals	intravenous, via left atrium, intracoronary, various doses 0.1 - 3 µg/kg/min, or 0.01 - 1 µg/kg	none
Su *et al.*, 1973	isolated right atria, isolated papillary muscles	30 pM – 30 µM	none none

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

d) RABBIT

Authors	Preparation	$[PGF_{2\alpha}]$ / dose	Inotropic response?
Nutter & Ratts, 1973	isolated left atria	28 µM	none
El Zayat *et al.*, 1977	isolated perfused heart	5.2 – 8.4 µmoles bolus	positive

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

e) CAT

Authors	Preparation	$[PGF_{2\alpha}]$	Inotropic effect?
Su *et al.*, 1973	isolated right atria, right ventricular papillary muscles	30 pM – 30 µM	none none
Ogletree *et al.*, 1975	isolated perfused hearts, papillary muscles	2.8 µM 280 pM – 2.8 µM	none none

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

f) CHICK

Authors	Preparation	$[PGF_{2\alpha}]$	Inotropic response?
Boroyan & Mirzoian, 1978	embryonic ventricular myocytes	2 µM	none

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

g) TOAD

Authors	Preparation	$[PGF_{2\alpha}]$	Inotropic response?
Nutter & Ratts, 1973	ventricular strips	28 μM	negative

Table I-1. Species dependent inotropic effects of $PGF_{2\alpha}$:

h) HUMAN

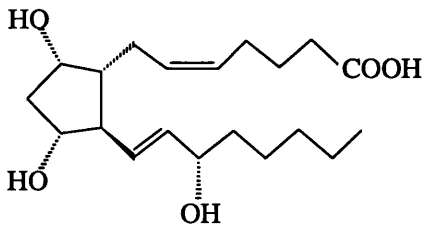
Authors	Subject	$PGF_{2\alpha}$ dose	Inotropic response?
Karim *et al.*, 1969	normal volunteers	intravenous infusion, 0.01-2 μg/kg/min	none, $PGF_{2\alpha}$ not detected in venous blood
Secher *et al.*, 1982	pregnant women	intravenous infusion, 100-300 μg/min (30 min)	increased cardiac work
Sziegoleit *et al.*, 1988	patients with cardiac extrasystoles	intravenous infusion, 25 – 100 μg/min	negative?, total electromechanical systole, preejection period and left ventricular ejection time prolonged

1.6 $PGF_{2\alpha}$ receptor

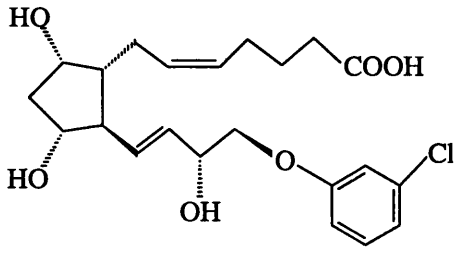
$PGF_{2\alpha}$ is an agonist for the FP prostaglandin receptor, which is coupled to G_q-protein activated phosphoinositide breakdown (reviewed in Coleman *et al.*, 1994). Since DNA and mRNA encoding the FP receptor are present in rat heart tissue (Lake *et al.*, 1994; Adams *et al.*, 1996), the positive inotropic effect of $PGF_{2\alpha}$ may be receptor mediated. Moreover, FP receptor mRNA is expressed in neonatal rat hearts primarily in ventricular myocytes and secondarily in smooth muscle (Adams *et al.*, 1996), suggesting that $PGF_{2\alpha}$ has a regulatory function in the myocardium. While the cloned FP receptor from the rat corpus luteum consists of 366 amino-acids, the cloned FP receptor from bovine corpus luteum and human uterus consist of 362 and 359 amino-acids respectively (Lake *et al.*, 1994; Sakamoto *et al.*, 1994; Abramovitz *et al.*, 1994). Since additional transcripts of lower size to that of the receptor were detected, either subtypes of the FP receptor exist in rat heart (Lake *et al.*, 1994), or the transcript encodes for a negative regulatory protein termed prostaglandin $F_{2\alpha}$ receptor associated protein, FPRP (Orlicky, 1996).

While $PGF_{2\alpha}$ is a potent ligand for the FP receptor, it is not selective since it can also bind the PGE selective EP, and thromboxane selective TP receptors. Conversely, the FP receptor can also be activated by PGD_2. Nevertheless, the availability of the highly selective FP receptor agonist, cloprostenol, allows FP receptor activation to be studied in the absence of potent FP receptor antagonists (Coleman *et al.*, 1994). Thus, in this thesis, initial experiments in the isolated perfused rat heart compares the positive inotropic effect induced by $PGF_{2\alpha}$ and cloprostenol. The chemical structures of $PGF_{2\alpha}$ and cloprostenol are shown in diagram I-6 below.

Diagram I-6. Chemical structures of $PGF_{2\alpha}$ and cloprostenol



$PGF_{2\alpha}$



cloprostenol

1.7 $PGF_{2\alpha}$ signal transduction mechanisms

Although the signal transduction mechanisms activated by $PGF_{2\alpha}$ have been studied in other cell types, there is little information regarding this in cardiac muscle (diagram I-7). The following sections will discuss the possible second messenger systems activated by $PGF_{2\alpha}$ and also its potential ways for Ca^{2+} modulation and Ca^{2+} sensitisation of the myofilaments.

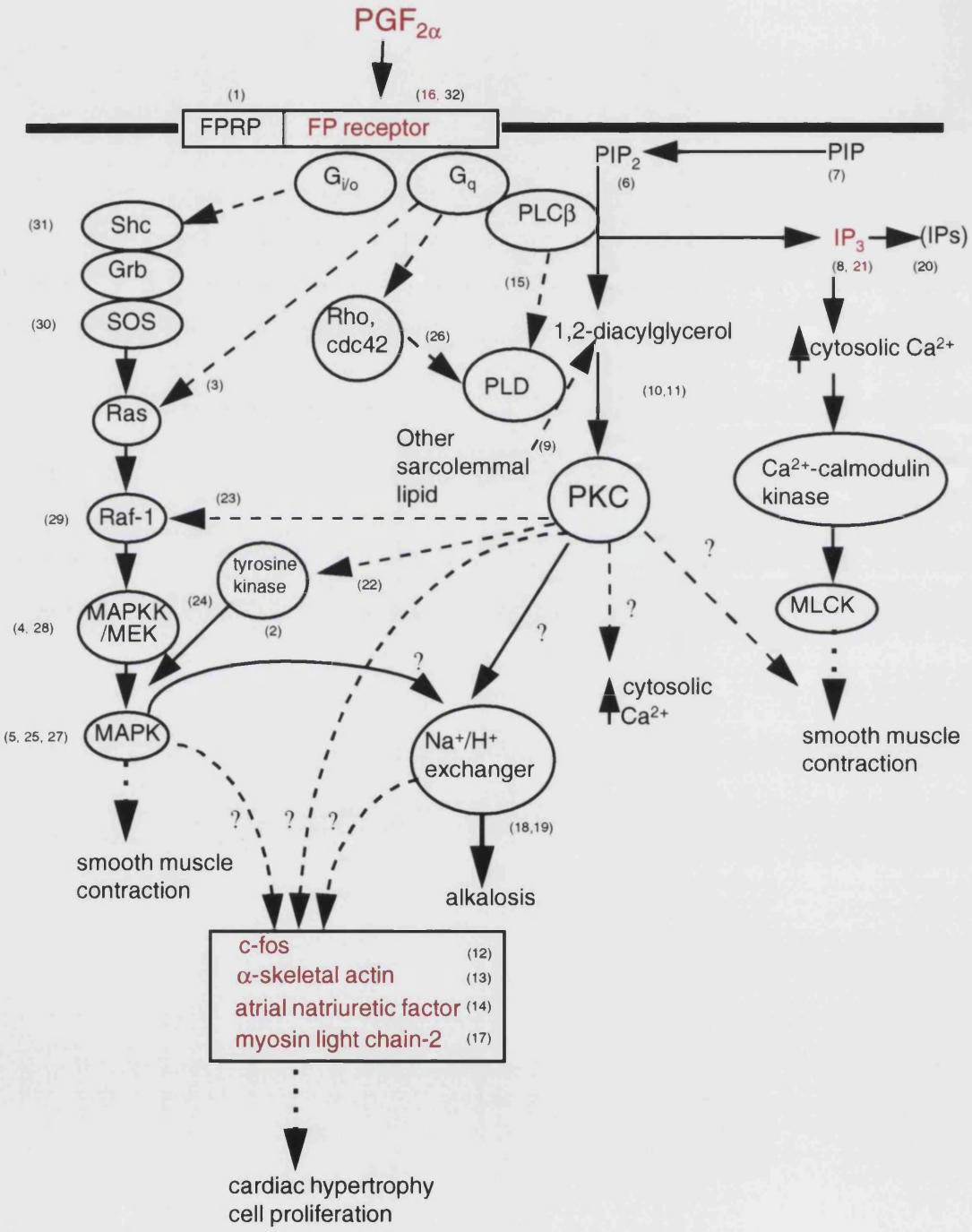
a) cAMP

Many positive inotropic agents increase cardiac contractility by increasing cytosolic levels of cAMP. Agents that activate the β_1-adrenergic receptor such as isoprenaline and noradrenaline, and agents that stimulate adenylate cyclase such as forskolin, and cAMP phosphodiesterase inhibitors such as milrinone, do increase cAMP and enhance cardiac contractility. cAMP activates protein kinase A, which phosphorylates L-type Ca^{2+} channels and phospholamban. This potentiates Ca^{2+} influx, and sarcoplasmic reticulum Ca^{2+} uptake and release, respectively. However, cAMP does not seem to be involved in the positive inotropic effect of $PGF_{2\alpha}$, as Otani *et al.* (1988) and Metsa-Ketela (1981) both showed that cAMP levels were unchanged in rat hearts treated with $PGF_{2\alpha}$.

b) Mitogen activated protein kinase (MAPK) cascade

The MAPK cascade is an important signalling pathway for cell proliferation. However, it may also regulate muscle contractility. In rat uterine muscle, Ohmichi *et al.* (1997) showed that rapid activation of this cascade is partially responsible for the $PGF_{2\alpha}$-induced contraction, as a MAPK kinase inhibitor reduced the contractile effect. Since $PGF_{2\alpha}$ also activates members of this cascade in NIH-3T3 cells (Watanabe *et al.*, 1995) and osteoblastic MC3T3-E1 cells (Hakeda *et al.*, 1997), there is a possibility that activation of this cascade plays a role in the positive inotropy of $PGF_{2\alpha}$ in the heart.

Diagram I-7. $PGF_{2\alpha}$ signal transduction mechanisms



Legend

The legend indicates where the receptor/second messenger was found, the concentration and period of contact with $PGF_{2\alpha}$, references, and other relevant details.

Experiments were done in cardiac tissue where highlighted red in the diagram.

——→ production or direct activation
·····→ indirect/(direct?) activation

1. rat pregnant corpus, Orlicky, 1996.

2. NIH-3T3 cells, 1- 3 min with 1 μM $PGF_{2\alpha}$, independent of extracellular Ca^{2+}, Ca^{2+}/calmodulin kinase, dependent on Ca^{2+}, Watanabe *et al.*, 1994.

3. NIH-3T3 cells, 3 min with 1 μM $PGF_{2\alpha}$, Watanabe *et al.*, 1995.

4. NIH-3T3 cells, peak at 3 min with 1 μM $PGF_{2\alpha}$, ED_{50} = 10 nM, independent of extracellular Ca^{2+}, Ca^{2+}/calmodulin kinase, Watanabe *et al.*, 1995.

5. NIH-3T3 cells, peaked at 3 min with 1 μM $PGF_{2\alpha}$, ED_{50} = 10 nM, partially dependent on Ca^{2+}. Dose dependence of MAPK & MAPKK activation similar to that of phospholipase C activation, Watanabe *et al.*, 1995.

6. Balb/c/3T3 fibroblast cells, breakdown within 1 min, Fukami & Takenawa, 1989.

7. Balb/c/3T3 fibroblast cells, breakdown within 7 min, Fukami & Takenawa, 1989.

8. Balb/c/3T3 fibroblast cells, max at 30 s, decreased to control level within 2 min, Fukami & Takenawa, 1989.

9. MC3T3-E1 osteoblast-like cells, 2 - 4 min with 1 μM $PGF_{2\alpha}$, dependent on Ca^{2+}, independent of PKC, partially responsible for 2nd DAG phase after 2 min from breakdown of other lipids, Sugiyama *et al.*, 1994.

10. MC3T3-E1 osteoblast-like cells, biphasic, Sugiyama *et al.*, 1994.

11. Balb/c/3T3 fibroblast cells, biphasic, 1st phase 30s, 2nd phase up to 7 min, Fukami & Takenawa, 1989.

12. neonatal myocytes, 30 min with 1 μM $PGF_{2\alpha}$, Lai *et al.*, 1996.

13. neonatal myocytes, 48 hr with 1 μM $PGF_{2\alpha}$, Lai *et al.*, 1996.

14. neonatal myocytes, 48 hr, 1 nM - 1 μM $PGF_{2\alpha}$, adult myocytes, 72 hr, 1 nM-1 μM $PGF_{2\alpha}$, Lai *et al.*, 1996.

15. MC3T3-E1 osteoblast-like cells, Sugiyama *et al.*, 1994.

16. neonatal rat ventricular myocytes > vascular smooth muscle cells >> cardiac fibroblast-like cells, Adams *et al.*, 1996.

17. neonatal rat ventricular myocytes, 36 hrs, 0.1 μM $PGF_{2\alpha}$, Adams *et al.*, 1996.

18. rat mesangial cells, 1 μM $PGF_{2\alpha}$, Mene *et al.*, 1991.

19. neonatal mouse clonal osteoblast-like cell line, MOB 3-4, 0.05 - 5 μg/ml $PGF_{2\alpha}$, Kawase *et al.*, 1991.

20. neonatal mouse clonal osteoblast-like cell line MOB 3-4, >> 50 ng/ml $PGF_{2\alpha}$, Kawase *et al.*, 1991.

21. rat papillary muscles, peaked at 30 s with 1 μM $PGF_{2\alpha}$, Otani *et al.*, 1988.

22. osteoblastic MC3T3-E1 cells, occurs within 5 min, max at 0.3 μM $PGF_{2\alpha}$, Hakeda *et al.*, 1997.

23. osteoblastic MC3T3-E1 cells, Hakeda *et al.*, 1997.

24. osteoblastic MC3T3-E1 cells, Hakeda *et al.*, 1997.

25. osteoblastic MC3T3-E1 cells, max at 2min with 1 μM $PGF_{2\alpha}$, PKC dependent, Hakeda *et al.*, 1997.

26. osteoblastic MC3T3-E1 cells, 1 μM $PGF_{2\alpha}$, 10 min, 37°C, Kato *et al.*, 1997.

27. rat puerperal uterine cells, increased activity after 5 min with 1 μM $PGF_{2\alpha}$, Ohmichi *et al.*, 1997.

28. rat puerperal uterine cells, 5 min with 1 μM $PGF_{2\alpha}$, , Ohmichi *et al.*, 1997.

29. rat puerperal uterine cells, 5 min with 1 μM $PGF_{2\alpha}$, , Ohmichi *et al.*, 1997.

30. rat puerperal uterine cells, occurs within 1 min with 1 μM $PGF_{2\alpha}$, maximum by 30 min, Ohmichi *et al.*, 1997.

31. rat puerperal uterine cells, 5 min with 1 μM $PGF_{2\alpha}$, Ohmichi *et al.*, 1997.

32. rabbit jugular vein, Chen *et al.*, 1995.

c) Phosphoinositide cascade

$PGF_{2\alpha}$ activates phospholipase C mediated phosphatidylinositol 4,5-bisphosphate (PIP_2) breakdown in a fibroblast cell line (Fukami & Takenawa, 1989). While increased levels of the breakdown product, inositol 1,4,5-trisphosphate (IP_3), are detected in rat papillary muscle following $PGF_{2\alpha}$ treatment (Otani *et al.*, 1988), sustained activation of the other breakdown product, 1,2-diacylglycerol, occurs in osteoblast-like and fibroblast cell lines (Sugiyama *et al.*, 1994; Fukami & Takenawa, 1989). Breakdown of other lipids such as phosphatidylcholine by phospholipase D may also contribute to this sustained diacylglycerol signal (Sugiyama *et al.*, 1994).

Both the PIP_2 breakdown products IP_3 and diacylglycerol may increase cardiac contractility by different mechanisms (De Jonge *et al.*, 1995). Although IP_3-induced release of Ca^{2+} from Ca^{2+} stores has been shown in many cell types (Berridge & Irvine, 1984), this may not occur in cardiac cells. Photorelease of 'caged IP_3' causes a small, inconsistent release of sarcoplasmic reticulum Ca^{2+} in saponin skinned ventricular trabeculae from rats (Kentish *et al.*, 1990). IP_3 does not release Ca^{2+} in rat ventricular myocytes or from canine cardiac sarcoplasmic reticulum vesicles (Movsesian *et al.*, 1985). Additionally, receptors for IP_3 in rat ventricular myocytes are not immunolocalised at the sarcoplasmic reticulum, but at the intercalated discs (Kijima *et al.*, 1993). In contrast, diacylglycerol increases cardiac contractility by activation of protein kinase C (Pi *et al.*, 1997).

d) Protein kinase C (PKC)

PKC is composed of a family of serine/threonine kinases that vary in their cofactor requirements for enzymatic activity, tissue distribution and subcellular localisation (Nishizuka, 1995; Hofmann, 1997). The inotropic effects of PKC are controversial, as PKC is claimed to increase or decrease contractility, either by modulating Ca^{2+} levels (Macleod & Harding, 1991; Capogrossi *et al.*, 1990; Karmazyn *et al.*, 1990), or by phosphorylating contractile proteins (Clement *et al.*, 1992; Jideama *et al.*, 1996). Furthermore, eleven isozymes of protein kinase C have been identified, and preferential activation of an isozyme may influence contractility. The current view is that in adult ventricular myocytes, PKC-ε is abundantly expressed (Bogoyevitch *et*

al, 1993; Rybin & Steinberg, 1994), PKC-δ and perhaps -α and-ζ are detectable (Puceat *et al.*, 1994; Rybin *et al.*, 1997) but PKC-β and -γ are not detectable (Rybin & Steinberg, 1994). The phosphorylation of myosin light chain by PKC has been implicated in its ability to sensitise cardiac myofilaments to Ca^{2+} (Clement *et al.*, 1992; Venema *et al.*, 1993), but PKC-α, -δ and -ε can decrease the sensitivity of actomyosin MgATPase by selective phosphorylation of troponin I and T. In contrast, PKC-ζ phosphorylation of troponin T results in a slight increase in Ca^{2+} sensitivity without affecting the maximal activity of MgATPase (Jideama *et al.*, 1996).

The modulation of cardiac membrane targets by PKC is controversial and is reviewed by Puceat and Vassort (1996). The potential phosphorylation sites and actions of PKC are listed in table I-2 below.

Table I-2. Modulation of cardiac membrane targets by PKC

Target	Action
15kDa protein named phospholemman	may or may not stimulate chloride conductance
phospholamban	increase or decrease sarcoplasmic reticulum Ca^{2+} uptake
K^+ channels: voltage activated outward, delayed rectifier	prolongation or shortening of the action potential, affecting the duration of Ca^{2+} influx
(Sna-rH1) Na^+ channel	decreases Na^+ current
Ca^{2+} channel	transient increase in L-type Ca^{2+} current, inhibition of T-type Ca^{2+} current
Na^+/H^+ exchanger	may or may not activate
Na^+/Ca^{2+} exchanger	may activate

1.8 Possibility of cytosolic Ca^{2+} level regulation by $PGF_{2\alpha}$

As discussed from the previous section, $PGF_{2\alpha}$ may increase intracellular Ca^{2+} via PKC or by IP_3. However, evidence for the role of Ca^{2+} in the $PGF_{2\alpha}$-induced positive inotropic effect is unclear.

$PGF_{2\alpha}$ is reported to accumulate $^{45}Ca^{2+}$ in the rat heart (Metsa-Ketela, 1981) but not in the guinea-pig heart (Sabatini-Smith, 1970). If Ca^{2+} accumulation does occur with $PGF_{2\alpha}$, a source of Ca^{2+} entry may be the L-type Ca^{2+} channel. Otani *et al.* (1988) reported that $PGF_{2\alpha}$ initiated contractions of 25 mM K^+ depolarised, electrically paced rat papillary muscles, and that this effect was inhibited with the L-type Ca^{2+} channel blocker, nifedipine. Furthermore, action potential duration is prolonged in rat and guinea-pig papillary muscles treated with $PGF_{2\alpha}$ (Forster *et al.*, 1974; January & Schottelius, 1974). One of the possible explanations for this effect is an increase in the L-type Ca^{2+} current, but this effect can also be interpreted either as a reduction in the K^+ and Cl^- currents, or an increase in the Na^+ current. However, the Ca^{2+} channel blocker, verapamil, does not inhibit the $PGF_{2\alpha}$-induced increase in guinea-pig cardiac contractility (Otani *et al.*, 1986).

$PGF_{2\alpha}$ also increases Ca^{2+} uptake of guinea-pig fragmented sarcoplasmic reticulum membranes (Sabatini-Smith, 1970), while apparent inhibition of sarcoplasmic reticulum Ca^{2+} release in guinea-pig atria reduces the $PGF_{2\alpha}$-induced positive inotropy (Otani *et al.*, 1986). Additionally, increased Ca^{2+} influx may occur by Na^+/Ca^{2+} exchange. Since $PGF_{2\alpha}$ inhibits the Na^+/K^+ ATPase activity of fragmented rat sarcolemmal membranes (Karmazyn *et al.*, 1981), this would in turn affect Na^+/Ca^{2+} exchange.

Thus, in this thesis, to find out if $PGF_{2\alpha}$ increases Ca^{2+} availability to the contractile filaments, I monitored Ca^{2+} transients by fluorimetric methods in electrically stimulated, fura-2 loaded rat ventricular myocytes. Additionally, Dr. Katherine Reeves monitored L-type Ca^{2+} currents in patch clamp experiments in the same laboratory.

1.9 Possibility of cytosolic pH regulation by $PGF_{2\alpha}$

PKC (Kandasamy *et al.*, 1995), members of the MAPK cascade (Noel & Pouyssegur, 1995) and Ca^{2+}-calmodulin kinase (Fliegel *et al.*, 1992; Le Prigent *et al.*, 1997) modulate intracellular pH by activating the sarcolemmal Na^+/H^+ exchanger in many cell types. The Na^+/H^+ exchanger extrudes one H^+ in return for one Na^+, and is driven by the Na^+ gradient generated by the Na^+/K^+ ATPase. It is an important mechanism for modulating internal pH, alongside the Na^+/HCO_3^- symport and the Na^+ dependent HCO_3^-/Cl^- antiport. Additionally, the Na^+/H^+ exchanger modulates cell growth, volume and salt balance (Noel & Pouyssegur, 1995).

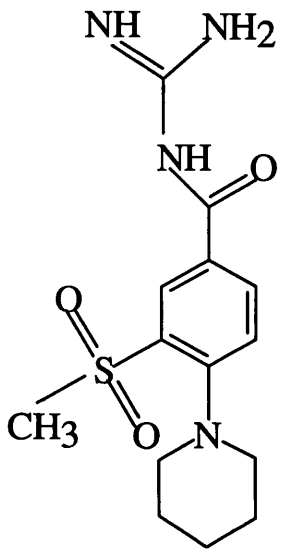
$PGF_{2\alpha}$ stimulates the Na^+/H^+ exchanger in kidney and osteoblast cell lines (Kawase *et al.*, 1991; Mene *et al.*, 1991). If such an effect also occurs in the $PGF_{2\alpha}$-treated heart, then the cytosolic alkalosis produced may be important in the positive inotropy. PKC activators such as phenylephrine and endothelin-1 are thought to increase cardiac contractility at least partly by activating the Na^+/H^+ exchanger and increasing cytosolic pH (Kramer *et al.*, 1991; Puceat *et al.*, 1992; Khandoudi *et al.*, 1994). This is because cytosolic alkalosis sensitises the contractile filaments to Ca^{2+} (Fabiato & Fabiato, 1978).

Cytosolic pH of myocytes can be measured by means of pH selective microelectrodes or by pH sensitive dyes (Frohlich & Wallert, 1995). In this thesis, the pH sensitive dye, BCECF, was used because of the availability of the instruments. Additionally, the involvement of the Na^+/H^+ exchanger in the positive inotropic effect of $PGF_{2\alpha}$ was tested by using the exchanger inhibitor, HOE 694 (Scholz *et al.*, 1993; diagram I-8). HOE 694 is more selective for the Na^+/H^+ exchanger compared to the older generation amiloride derivatives. It also preferentially inhibits NHE-1 (which stands for Na^+/H^+ isoform 1), which is predominant in the heart, over the other isoforms NHE-2, NHE-3 and NHE-4 (Loh *et al.*, 1996).

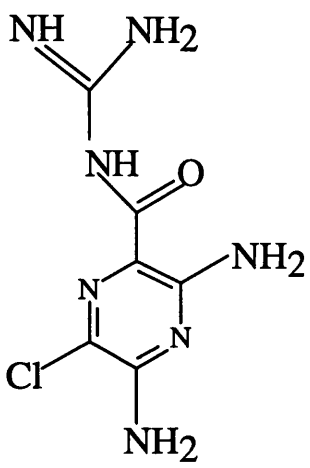
In addition, the potent and selective inhibitor of PKC, chelerythrine, was used in this thesis to investigate the role of PKC in mediating the effects of $PGF_{2\alpha}$. Chelerythrine is an alkaloid that inhibits PKC with an IC_{50} of 0.66 μM (Herbert *et al.*, 1990;

diagram I-8). It interacts with the catalytic domain of PKC at a site different from that of phorbol esters. Unlike the other classical PKC inhibitors such as staurosporine and H7 that inhibit other protein kinases, very high concentrations of chelerythrine are needed to inhibit protein kinase A, tyrosine kinase or Ca^{2+}/calmodulin protein kinase (Herbert *et al.*, 1990).

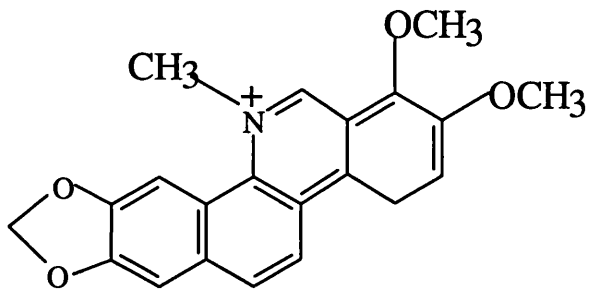
Diagram I-8. Chemical structures of the Na^+/H^+ inhibitors HOE 694 and amiloride, and the PKC inhibitor, chelerythrine.



HOE 694



amiloride



chelerythrine

1.10 Aims of this study

1. To characterise the effects of $PGF_{2\alpha}$ in Langendorff perfused, non-working rat hearts, and to ascertain whether similar effects could be induced via FP receptor stimulation using the selective FP agonist, cloprostenol.

2. To characterise the positive inotropic effect of $PGF_{2\alpha}$ in paced left atria of rats.

3. To characterise the mechanism of action of $PGF_{2\alpha}$ at the cellular level using rat ventricular myocytes.

SECTION 2

Materials and Methods

2.1 Animals

Male Wistar rats of 280 - 310g were supplied by the University of Bath Animal House.

2.2 Drugs and chemicals

Drug	Source	Stock and storage
BCECF/AM	Calbiochem (Nottingham)	dissolved in anhydrous DMSO to 5 mM stock and stored at -20°C
chelerythrine chloride	Calbiochem	dissolved in 1:4 dimethyl sulphoxide:water to 10 mM and stored at -20°C
cloprostenol	gift from Dr. Robert Coleman of Glaxo	dissolved in ethanol to 250 µg/ml (5.9×10^{-4} M) and stored at 4°C
collagenase (type II)	Worthington Biochemical Corporation (New Jersey)	4°C
fura-2/AM	Calbiochem	dissolved in anhydrous DMSO to 5 mM stock and stored at -20°C
HOE 694	gift from Dr. H.-J. Lang of Hoechst Pharmaceuticals	dissolved in saline to 10 mM and stored at -20°C
laminin	Sigma Chemical Company (Poole)	made up to 15-20 µg/ml in KR buffer and stored at -20°C
nigericin	Sigma Chemical Company	dissolved in 95% ethanol to 10 mM and stored at -20°C
(-) - noradrenaline bitartrate salt	Sigma Chemical Company	dissolved in saline & 1 mM ascorbic acid before use

Drug	Source	Stock and storage
prostaglandin $F_{2\alpha}$ tromethamine salt	ICN Pharmaceuticals (Thame) & Sigma Chemical Company	dissolved in 7 parts ethanol and 3 parts water to 10 mM and stored at -20°C
protease (type XIV)	Sigma Chemical Company	-20°C

Analytical grade chemicals for buffers were purchased from Fisons Scientific Equipment (Loughborough) and British Drug Houses (BDH, Poole).

2.3 Buffers and solutions

2.3.1 Modified Krebs-Henseleit buffer

For:

a) Langendorff perfused hearts

b) isolated atrial preparations

Composition in mM: NaCl 118, D-Glucose 11.6, $NaHCO_3$ 25, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.23, gassed with 95 % O_2 : 5 % CO_2.

2.3.2 Modified Krebs-Ringer buffer

For:

a) myocyte isolation

b) fluorescent probe loading

c) single myocyte experiments

Composition in mM: NaCl 118.5, $NaHCO_3$ 14.5, KCl 2.6, KH_2PO_4 1.2, $MgSO_4$ 1.2, D-Glucose 11.1, HEPES 10.0, $CaCl_2$ 1.0, made up to pH 7.4 with NaOH for single myocyte experiments.

When this buffer was used for dissociating myocytes, it was gassed with 95 % O_2 : 5 % CO_2, and $CaCl_2$ was excluded or added in nominal amounts (see 2.6.1).

When this buffer was used for loading myocytes with fura-2/AM or BCECF/AM, it was supplemented with 0.5 % bovine serum albumin and Ca^{2+} was omitted.

2.3.3 High K^+-nigericin solution

For:

a) myocyte pH_i calibration

Composition in mM: KCl 140, MgCl 1, HEPES 20, nigericin 0.01, made up to pH 6.8, 7.2 and 7.6 with NaOH.

2.4 Isolated perfused rat hearts

This model was used to compare the effects of $PGF_{2\alpha}$ and the selective FP receptor agonist, cloprostenol, on contractility, heart rate and coronary tone.

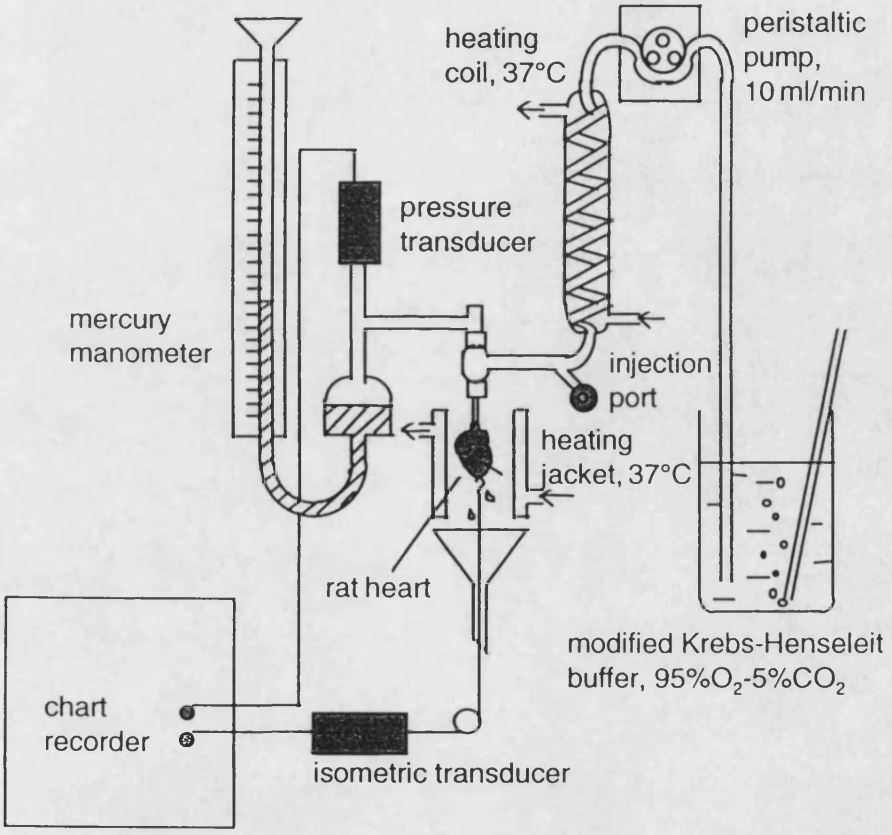
2.4.1 Experimental set up

Rats were anaesthetised with intraperitoneal 100 mg/kg pentobarbitone sodium and killed by cervical dislocation. The heart was excised with the aorta intact and immediately immersed in ice-cold modified Krebs-Henseleit buffer (see 2.3.1). As shown in diagram M-1 (see 2.4.3), the aorta was then cannulated so that the heart was perfused through its own circulation with buffer under constant flow conditions of 10 ml/min at 37°C. The buffer was delivered using a Watson-Marlow flow inducer. A 25-gauge needle was inserted into the chamber of the left ventricle to drain the perfusion fluid flowing from the Thebesian vessels. Coronary perfusion pressure was monitored by a pressure transducer connected to the aortic cannula. Developed tension was measured under 2 g resting tension by means of a hook, piece of thread and pulley aligned in a single vertical plane attached to the apex of the left ventricle at one end and an isometric transducer at the other end. Signals from the isometric transducer also triggered a heart rate meter. Traces were recorded with either Gould 2400S or Gould RS3400 recorders. Bolus doses of not more than 100 µl of drugs were introduced through an injection port near the aortic cannula.

2.4.2 Measurements

The contractile response was measured as the difference between the maximum developed tension and resting tension, defined as developed tension. Inotropic responses are shown as the percentage increase in developed tension. Changes in heart rate are stated in beats per minute while changes in perfusion pressure are indicated in millimetres of mercury (mmHg).

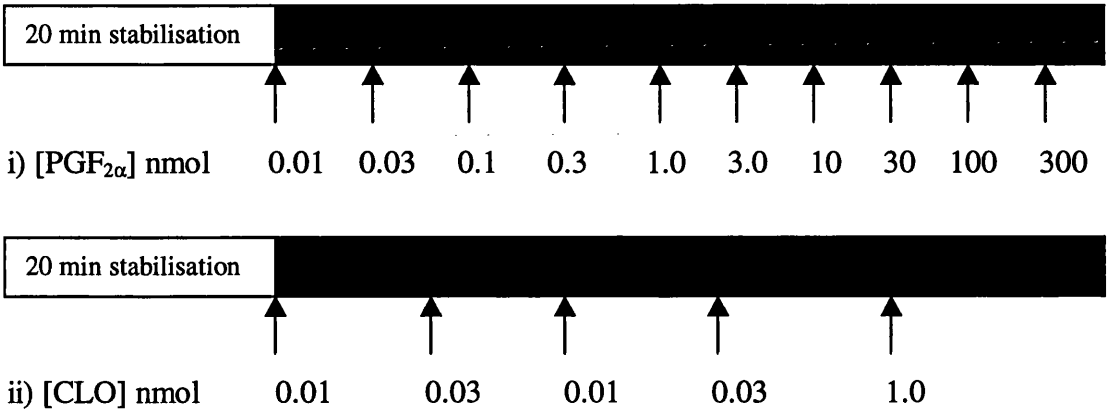
2.4.3 Diagram M-1. Schematic representation of the isolated perfused rat heart preparation



2.4.4 Protocols

Dose dependent actions of $PGF_{2\alpha}$ and cloprostenol (CLO) on contractility, heart rate and coronary tone

Bolus doses of $PGF_{2\alpha}$ or cloprostenol were introduced after the effects of the previous dose had worn off.



2.5 Isolated rat left atria

The positive inotropic effect of $PGF_{2\alpha}$ was characterised in the absence of perfusion pressure changes in electrically stimulated atria. This positive inotropy was also compared to that of noradrenaline.

2.5.1 Experimental set up

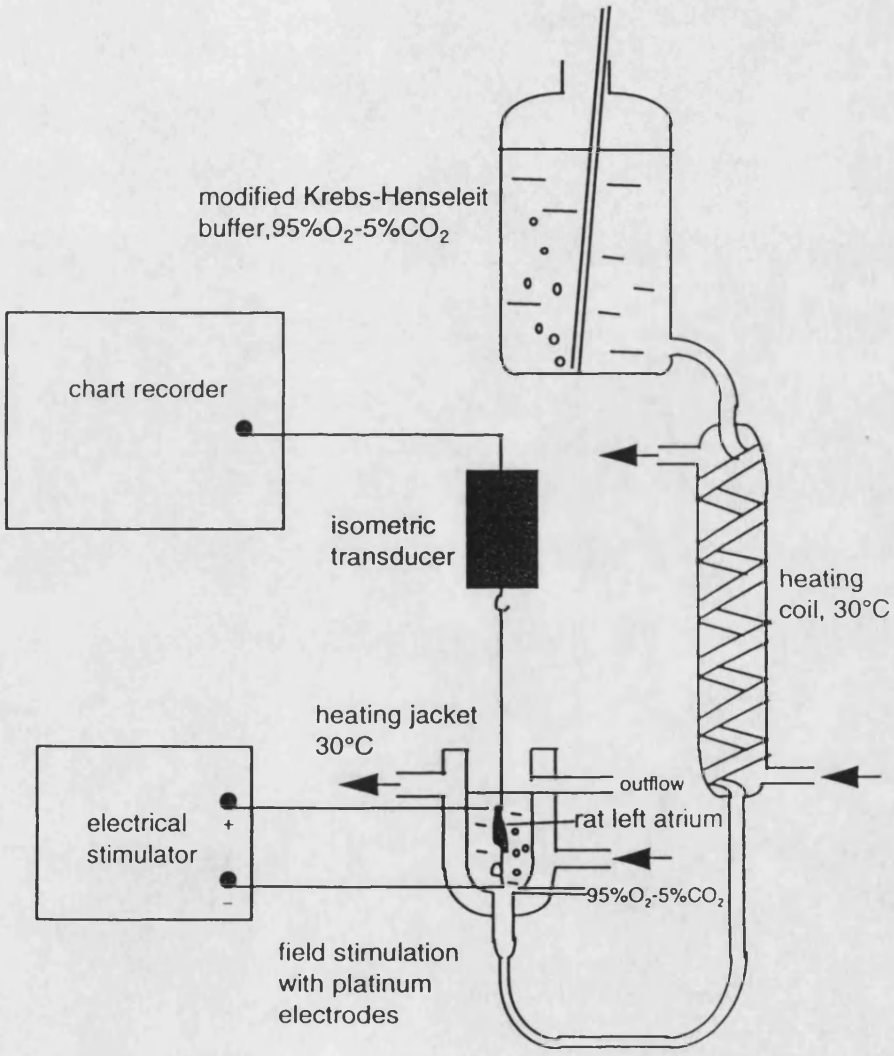
The left atrium was dissected from the isolated rat heart and mounted in a 10 ml organ bath containing modified Krebs-Henseleit buffer (see 2.3.1) at 30°C. As shown in diagram M-2 (see 2.5.3), one end of the atrium was tied to a platinum electrode, while the other end was tied to an isometric tension transducer. The atrium was field stimulated by platinum electrodes at 0.2 Hz, 1 ms pulse width at 20 - 40 V, under a resting tension of 500 mg. Developed tension was monitored with a Lectromed recorder connected to the isometric tension transducer. Drugs were injected into the organ bath with a Hamilton syringe.

2.5.2 Measurements

The contractile response was measured as the difference between developed tension and resting tension, defined as developed tension. Inotropic responses are shown as percentage increases in developed tension.

Effects on the rate of relaxation were quantified by comparing the ratio of the maximal velocity of contraction (+T) and maximal velocity of relaxation (-T), before and after $PGF_{2\alpha}$ treatment.

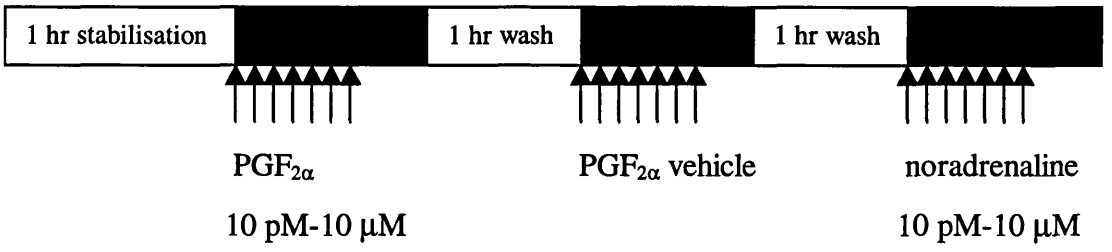
2.5.3 Diagram M-2. Schematic representation of the isolated rat left atrial preparation



2.5.4 Protocols

a) Effects of $PGF_{2\alpha}$ and noradrenaline on atrial developed tension

$PGF_{2\alpha}$, its vehicle, and noradrenaline were added into the organ bath when the response to the previous dose had stabilised. Addition of $PGF_{2\alpha}$ and noradrenaline were done in the reverse order in some experiments to compensate for the larger inotropic effect to drugs at the end of the experiment. In some cases, noradrenaline was added after the maximum increase in contractility was achieved with $PGF_{2\alpha}$, to see if the noradrenaline-induced increase in contractility was additive to that of $PGF_{2\alpha}$.



b) Effect of $PGF_{2\alpha}$ (10 μM) on the contraction-relaxation cycle duration

Chart speed was increased from 1 mm/min to 100 mm/s, and the contraction-relaxation cycle compared in the presence and absence of $PGF_{2\alpha}$.

2.6 Isolated rat ventricular myocytes

The contractile effects of $PGF_{2\alpha}$ were studied on enzymatically dissociated rat ventricular myocytes. Myocyte shortening was measured with a video image and edge detector system, while Ca^{2+} transients and pH_i were monitored spectrofluorimetrically in fura-2 and BCECF loaded myocytes respectively. The effects of the Na^+/H^+ exchanger inhibitor, HOE 694, and the PKC inhibitor, chelerythrine, on the $PGF_{2\alpha}$-induced increase in myocyte shortening and pH_i were also investigated.

2.6.1 Myocyte isolation

Calcium tolerant myocytes were prepared daily by a method reported by Powell *et al.* (1980). Some modifications to their technique were made. The perfusion system was primed with 50 ml Ca^{2+}-free modified Krebs-Ringer buffer (see 2.3.2), gassed with 95% O_2 and 5% CO_2. The isolated rat heart was cannulated and perfused for four minutes at 10 ml/min to clear the coronary vessels of blood. After that, the heart was perfused with modified Krebs-Ringer buffer containing 100 u/ml collagenase (type II), 0.5 u/ml protease (type XIV) and 20 μM Ca^{2+} for 15 minutes in a recirculating manner.

The digested heart was taken off the cannula, the aorta and atria discarded, and the ventricles sliced into four sections. The ventricular tissue was teased apart with forceps and gently shaken in 20 ml of gassed enzyme solution in a conical flask at 37°C for five minutes. The myocyte suspension was then filtered through a 100 μm nylon mesh gauze into a centrifuge tube and the filtrate centrifuged at 14 *g* for five minutes. The supernatant containing collagenase was removed and the myocyte pellet resuspended in Ca^{2+}-free buffer. This centrifugation and resuspension procedure was carried out twice. Ca^{2+} was gradually added to 1 mM to produce single Ca^{2+} tolerant myocytes.

2.6.2 Myocyte shortening experiments

2.6.2.1 Experimental set up

Single myocytes attached to a 0.1 ml volume laminin (15 μg/ml) coated perfusion chamber, were focused under a X 25 objective and the image projected by a camera to a video recorder and TV monitor (see 2.6.2.3, diagram M-3). The camera was rotated so that the myocyte length was vertical on the monitor. The edge detector system (vertical video length interface supplied by Richard Montgomery, National Heart and Lung Institute, London) was similar to that described by Harding *et al.* (1988). The edge detector was interposed between the video recorder and monitor, and it generated a movable rectangular window on the monitor. The window was positioned to enclose the myocyte image from the top to the bottom edges. The threshold of contrast between the myocyte edge and outer region was set so that the edge of the myocyte appeared white and the background enclosed by the window appeared black. The edge detection device monitored the number of television lines between the top of the screen and the first point of high contrast within the window (top myocyte edge), and from the top myocyte edge to the last point of contrast within the window (bottom myocyte edge). Hence, the length of the myocyte and myocyte shortening during electrical stimulation could be monitored when the device was connected to a chart recorder, and when connected by the RS232 port to the computer.

Only rod-shaped, striated, normally quiescent cells that responded to electrical stimulation with a rapid twitch were selected for single myocyte recordings. Myocytes were field stimulated at a rate of 1 Hz for 1 ms, at a voltage between 20 - 60 V, with silver chloride electrodes placed about 2 - 4 mm apart. They were superfused with 1 mM Ca^{2+} containing modified Krebs-Ringer buffer (see 2.3.2) at 2 ml/min. Experiments were done at room temperature (24 - 26°C).

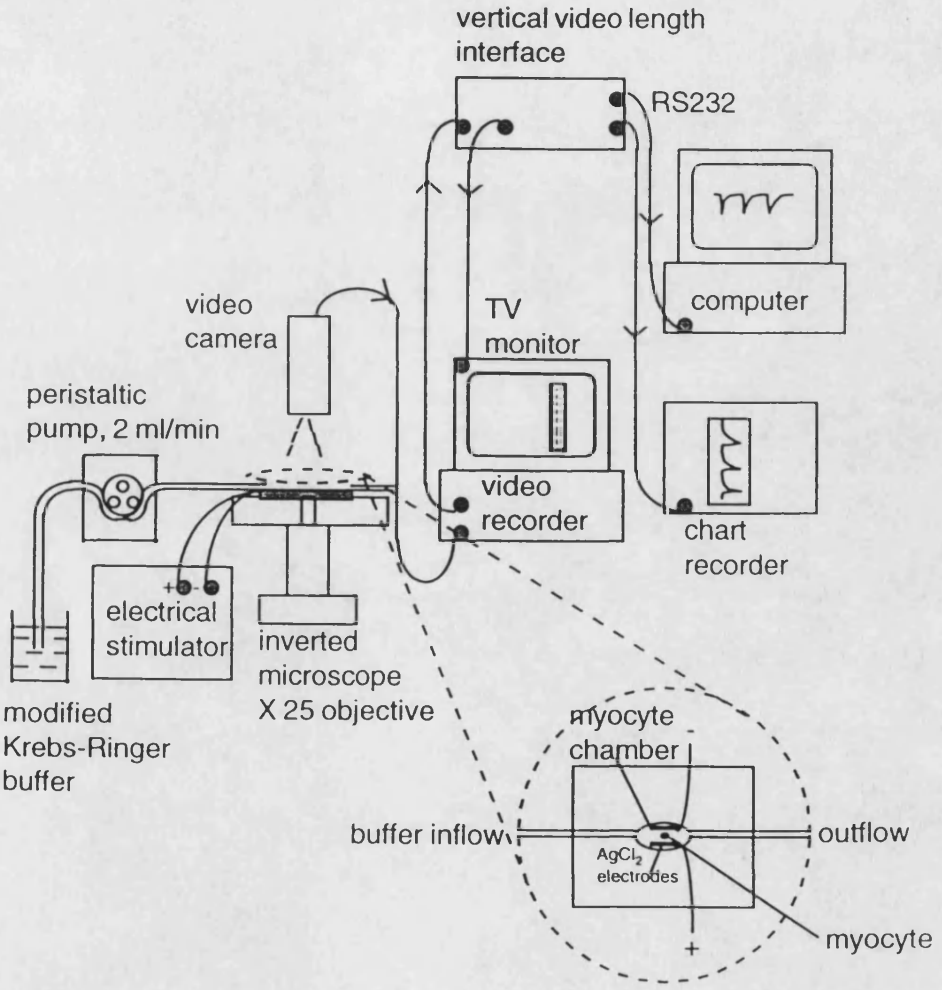
2.6.2.2 Measurements

Computer analysis of the data obtained at selected periods averaged myocyte shortening, time to peak shortening, and time to 50% and 90% relaxation over six

contractions. This was made possible with the computer programs provided by Dr. Peter O'Gara and Dr. Sian Harding (National Heart and Lung Institute, London). Myocyte length could also be calibrated by chart recorder measurements of graticule distances projected onto the monitor. Measurements obtained this way were similar to that of the computer measurements. The experiments were also recorded on video tape and could be played back through the edge detector device for analysis through the chart recorder or the computer.

Shortening of cardiac myocytes following drug treatment is expressed as the percentage difference in absolute shortening compared to before drug treatment. For example, if a 100 μm long myocyte shortened by 10 μm, and drug treatment increased the shortening to 15 μm, then the increase in shortening was expressed either as an increase of 50%, or 150% of control shortening. Six consequent shortening events are averaged at the stated time.

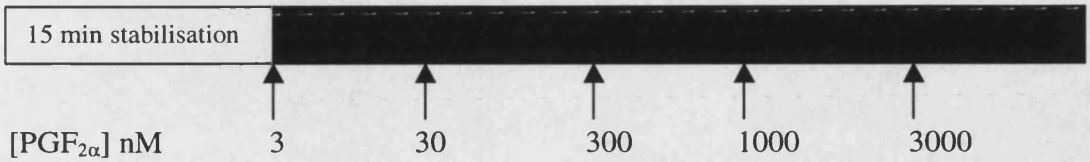
2.6.2.3 Diagram M-3. Schematic representation of the system for measuring myocyte shortening



2.6.2.4 Protocols

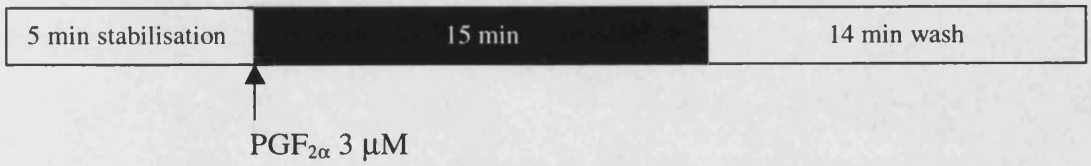
a) Effect of cumulative addition of $PGF_{2\alpha}$ on myocyte shortening

$PGF_{2\alpha}$ was added after the effect of the previous dose reached a plateau, usually after 10 minutes. Since 3 μM $PGF_{2\alpha}$ produced a large increase in myocyte shortening, this standard concentration was used in later studies.



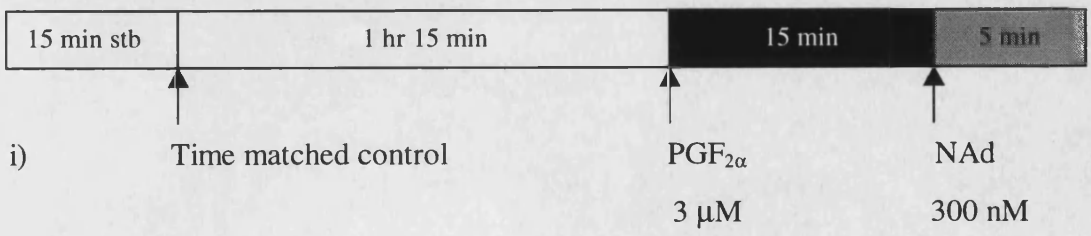
b) Effect $PGF_{2\alpha}$ on shortening of myocytes loaded with 5 μM fura-2

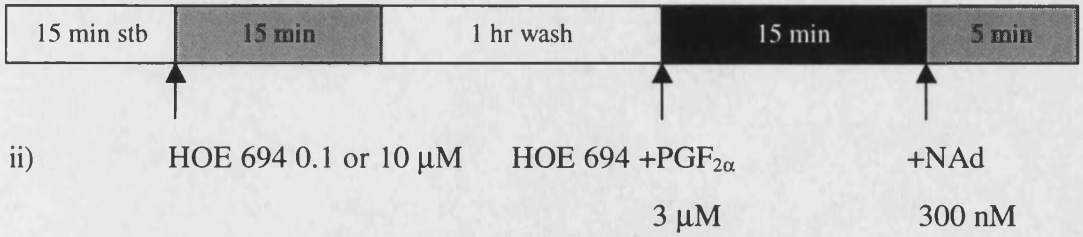
Since fura-2 can buffer intracellular Ca^{2+} (Noble & Powell, 1990), this set of experiments was performed to ensure that this concentration of fura-2 used was not affecting the $PGF_{2\alpha}$-induced increase in myocyte shortening. Data from these experiments were also used for construction of the phase-plane diagram (see 2.6.4.1).



c) Effects of the Na^+/H^+ exchanger inhibitor, HOE 694 (0.1 and 10 μM), on $PGF_{2\alpha}$-induced increases in myocyte shortening

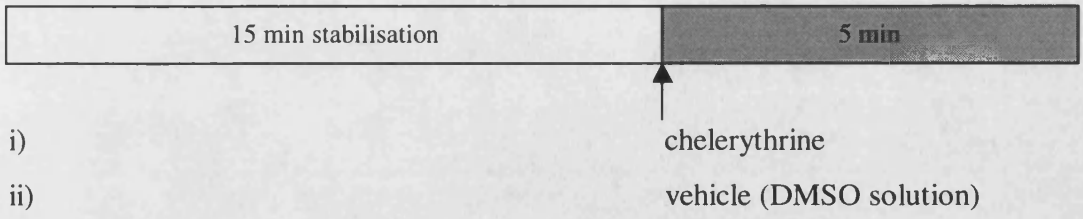
In preliminary experiments, 10 μM HOE 694 inconsistently affected basal shortening, therefore its basal effects were compared to when it was combined with $PGF_{2\alpha}$ in the same myocyte. Noradrenaline (NAd) was added at the end to ensure myocytes in all groups had a similar ceiling for shortening.



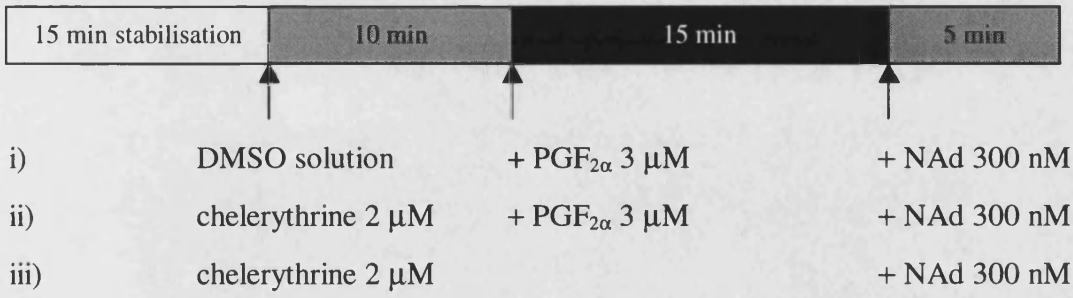


d) Effect of the PKC inhibitor, chelerythrine (2, 3, 5, 10 μM), on basal myocyte shortening

These preliminary experiments were done to characterise the contractile effects of chelerythrine. The chelerythrine concentration of 2 μM was chosen for later studies, based on the results obtained from these experiments. DMSO solution was used in protocol (ii) as a vehicle control for chelerythrine.



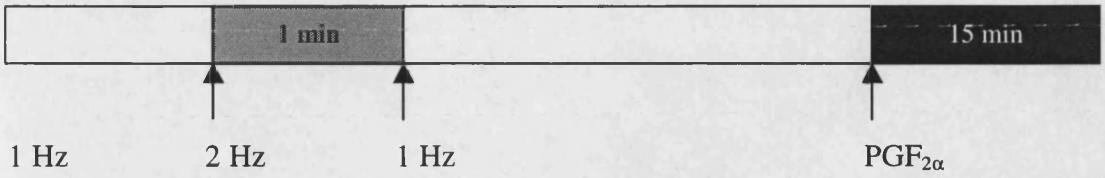
e) Effect of chelerythrine on $PGF_{2\alpha}$-induced increase in myocyte shortening



f) Effects of rapid pacing: relationship between the increase in contractility due to rapid pacing and that induced by $PGF_{2\alpha}$

With some batches of myocytes, prolonged stimulation at 1 Hz caused the myocyte under observation to develop a contracture, and therefore the experiment was terminated.

In order to identify better myocytes, myocytes were paced at 2 Hz for one minute at the beginning of an experiment. Those myocytes that could follow the pacing train, and survive this procedure, were usually capable of lasting for the duration of the experiment. The changes in myocyte shortening during rapid pacing and that with $PGF_{2\alpha}$ treatment were noted.



2.6.3 Ca^{2+} and pH_i fluorescence experiments

The advent of fluorescent agents that change their spectral properties upon binding the ion of interest such as Ca^{2+} (e.g. by fura-2 or indo-1), or H^+ (e.g. by BCECF or SNARF) allow the concentrations of these ions to be followed in the intact myocyte. In this study, fura-2 and BCECF were used to monitor intracellular Ca^{2+} and pH respectively. They are introduced into the myocyte as the hydrophobic acetoxymethyl (AM) ester form which passes through the sarcolemmal membrane. Once inside the myocyte, intracellular esterases cleave the ester, leaving the agent in the cytosol. The myocyte is illuminated with the appropriate excitation wavelength, fluorescence at the emission wavelength monitored, and the concentration of the ion calculated according to the calibrated fluorescence ratio.

With fura-2, binding of Ca^{2+} causes a hypsochromic shift (shift to a shorter wavelength) of the excitation spectra. As a result, fluorescence intensity increases at an excitation wavelength of 340 nm but decreases with excitation at 380 nm, when monitored at an emission wavelength of 510 nm (Grynkiewicz *et al.*, 1985). The signal ratio of 340/380 is linearly proportional to the concentration of the fluorescent species as fura forms a 1:1 complex with Ca^{2+}. The fura-2 transient arising from each electrical stimulation represents cytosolic free Ca^{2+}, and is dependent on all the processes contributing to, and removing Ca^{2+} from the cytosol as described earlier in section 1.3. Additionally, while allowing small changes in the Ca^{2+} level to be detected, the ratiometric method is reported to eliminate artifacts arising from total dye concentration, dye leakage, cell thickness, or absolute sensitivity of the instrument (Grynkiewicz *et al.*, 1985). Problems encountered with this method include photobleaching, compartmentation of dye into intracellular organelles and incomplete hydrolysis of ester groups producing Ca^{2+} insensitive intermediates.

The same principle is used with BCECF when measuring intracellular pH. The BCECF loaded myocyte is illuminated with excitation wavelengths of 440 and 490 nm. At the emission wavelength of 535 nm, the fluorescence intensity at the excitation wavelength of 490 nm increases with increasing pH, while that at 440 nm remains the same (isobestic point). Hence, the 490/440 ratio increases linearly with pH within the range of around pH 6.5 - 7.5 (Rink *et al.*, 1982). This linear

relationship is exploited in the calibration procedure, which involves perfusing the myocytes with high K^+ and nigericin (H^+/K^+ antiporter, Thomas *et al.*, 1979) containing calibration solutions of different pH values (see 2.3.3). Hence, the pH_i of each myocyte can be determined by a linear regression of the fluorescence ratio versus the pH value of the calibration buffer (Borzak *et al.*, 1990). The high potassium is required to eliminate membrane potential.

2.6.3.1 Fura-2/AM or BCECF/AM loading

Myocytes were loaded with the Ca^{2+} sensitive fluorescent dye, fura-2/AM, by incubating a 2.5 - 5 ml aliquot (about 1/20 of the myocyte yield) of myocytes with 5 μM fura-2/AM and 0.5 % bovine serum albumin in Ca^{2+} free Krebs-Ringer buffer (see 2.3.2). These myocytes were kept at 37°C for 30 minutes in an aluminium foil enclosed centrifuge tube. Removal of extracellular fura-2/AM and gradual exposure of the myocytes to Ca^{2+} was performed with three times centrifugation (14 *g*, 5 min) and resuspension with 0.1, 0.6 and 1 mM Ca^{2+}-containing modified Krebs-Ringer buffer. Similar procedures were carried out when the myocytes were loaded with the pH sensitive fluorescent dye, BCECF/AM, except that myocytes were loaded with 2 - 3 μM dye at room temperature for 20 minutes.

2.6.3.2 Experimental set up

Isolated myocytes, attached to a 0.1 ml volume laminin-coated perfusion chamber, were observed under a X 40 oil immersion objective on an inverted Nikon Diaphot 300 microscope in a darkened room. Only rod-shaped, striated, fluorescent, normally quiescent myocytes that responded to stimulation with a rapid twitch were selected for single myocyte recordings. These myocytes were field stimulated at a rate of 1 Hz for 1 ms, at a voltage between 20 - 80 V, with silver chloride electrodes placed about 2 - 4 mm apart. Experiments were done at room temperature (24 - 26°C), and myocytes were superfused with 1 mM Ca^{2+}-containing modified Krebs-Ringer buffer (see 2.3.2) at 2 ml/min.

Experiments were done with a Deltascan dual monochromator Photon Technologies International spectrofluorimeter system (see 2.6.3.4, diagram M-4).

Autofluorescence was first measured from a non-myocyte area similar in size to that of the selected myocyte. The viewed area on the photometer arm could be adjusted with movable slits. The fura-2 loaded myocyte was then brought into view and alternately excited with 340 and 380 nm wavelengths, and the fluorescence detected at the emission wavelength of 510 nm. The excitation light was reflected to the myocyte by a dichroic mirror placed under the objective, and the resulting fluorescence from the myocyte transmitted back to the dichroic mirror and towards the photomultiplier at the end of the photometer arm. Autofluorescence signals were then subtracted from the experimental signals, before data were analysed by Photon Technologies International computer software.

Measurements of pH_i were done in a similar way. BCECF loaded myocytes were electrically stimulated and alternately excited with 440 and 490 nm wavelengths. Fluorescence was detected at an emission wavelength of 535 nm. pH_i was calibrated for each myocyte, but whenever it was not possible to do so (e.g. myocyte washed away), calibrations were carried out with another myocyte from the same batch. Myocytes were perfused with a high K^+ and nigericin solution (see 2.3.3) of pH 6.8, pH 7.2, and pH 7.6, in random order.

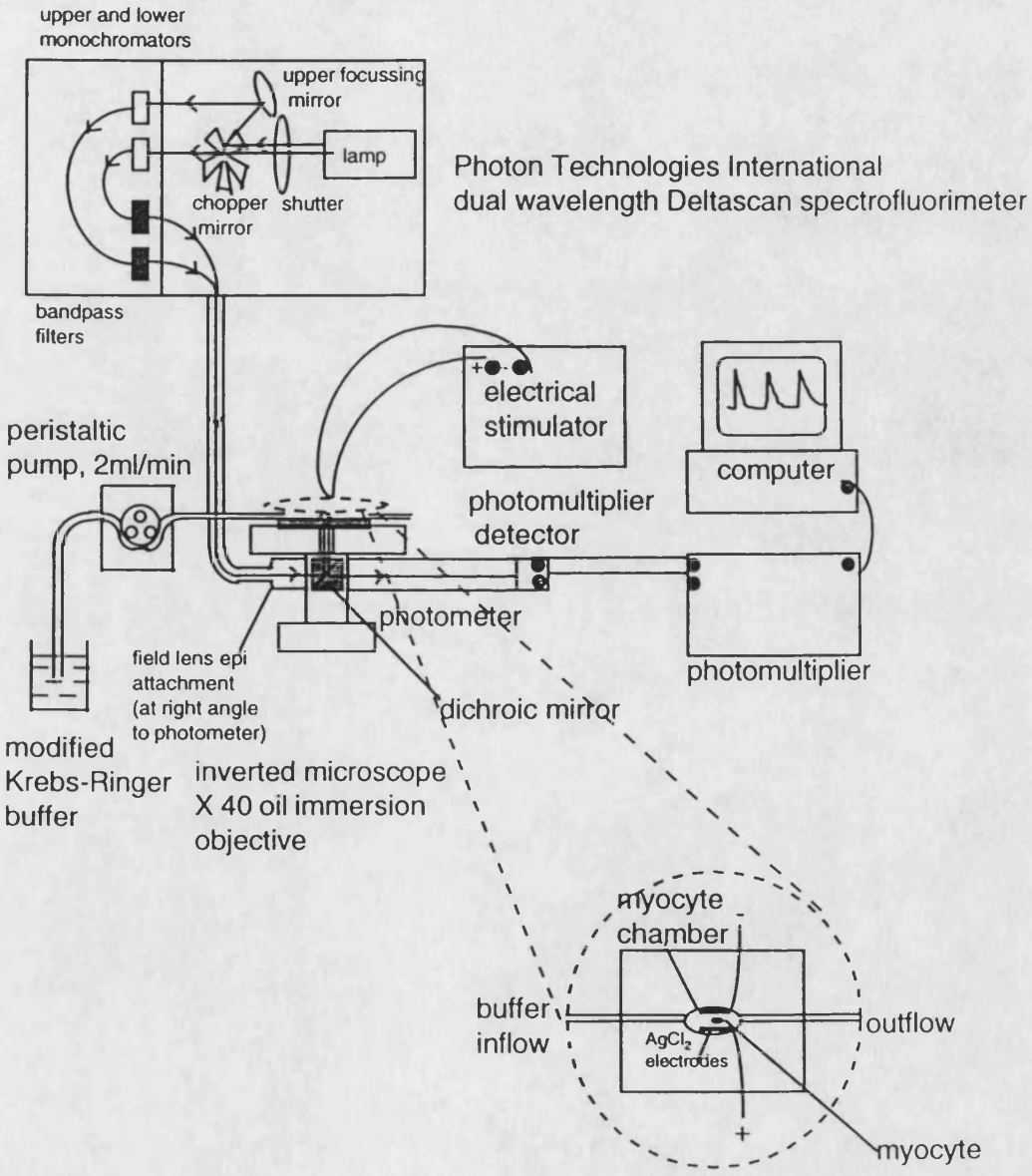
2.6.3.3 Measurements

Ca^{2+} measurements were obtained at 50 ms intervals. Diastolic fura-2 340/380 ratio represents diastolic Ca^{2+} level, while the amplitude of the 340/380 ratio spike represents the difference between the systolic and diastolic Ca^{2+} concentration. Ten Ca^{2+} transients before, and at the stated time after addition of a drug, were first averaged in each myocyte. These values were then averaged to those found with other cells.

The duration of the 340/380 ratio spike amplitude was measured at 5% of the amplitude value above the diastolic level. This value was first averaged over ten spikes for each myocyte, and then averaged for all myocytes. BCECF signals were only recorded for 10 s at 1 min intervals over the duration of the experiments to minimise photobleaching of BCECF. Signals were averaged to two points for every

3 s, and calibrated by linear regression of the 490/440 ratio versus the pH value of the calibration buffer.

2.6.3.4 Diagram M-4. Schematic representation of the system for measuring myocyte fluorescence



2.6.3.5 Protocols for Ca^{2+} transient experiments

a) Effects of noradrenaline on Ca^{2+} transients

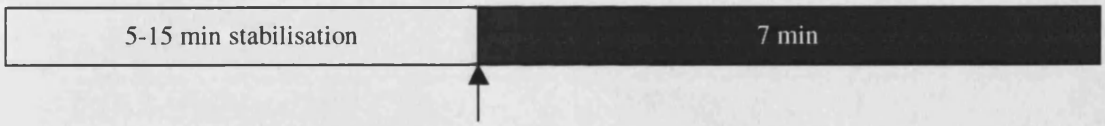
The effects of noradrenaline on Ca^{2+} transients were characterised so that it could be used as a positive control.

i) noradrenaline 300 nM

ii) vehicle

b) Effect of $PGF_{2\alpha}$ on Ca^{2+} transients

Ca^{2+} transients in the presence of $PGF_{2\alpha}$ were monitored with the protocol below. In some experiments, $PGF_{2\alpha}$ was washed out for 12 minutes, and noradrenaline was added for 100 seconds. Data from these experiments were also used to construct the phase-plane diagram (see 2.6.4.1).



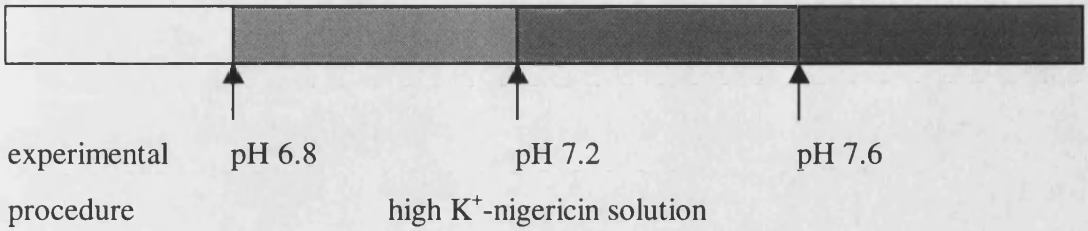
i) $PGF_{2\alpha}$ 3 μM

ii) vehicle

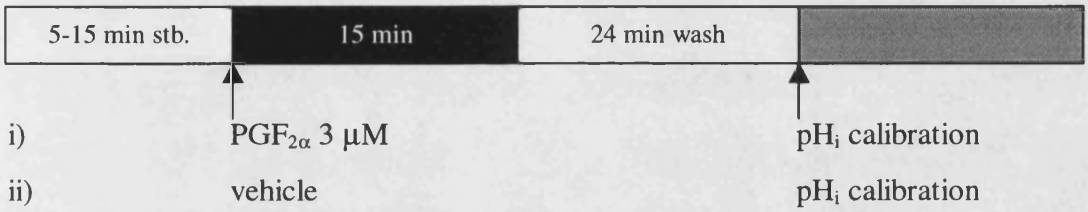
2.6.3.6 Protocols for pH_i experiments

a) pH_i calibration

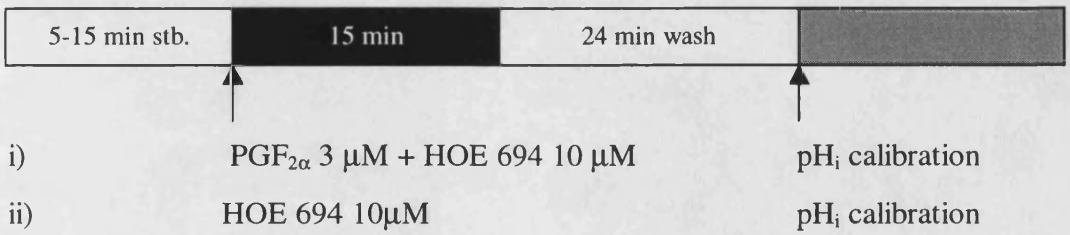
High K^+-nigericin solutions of different pH were added in random order and the BCECF fluorescence ratio recorded.



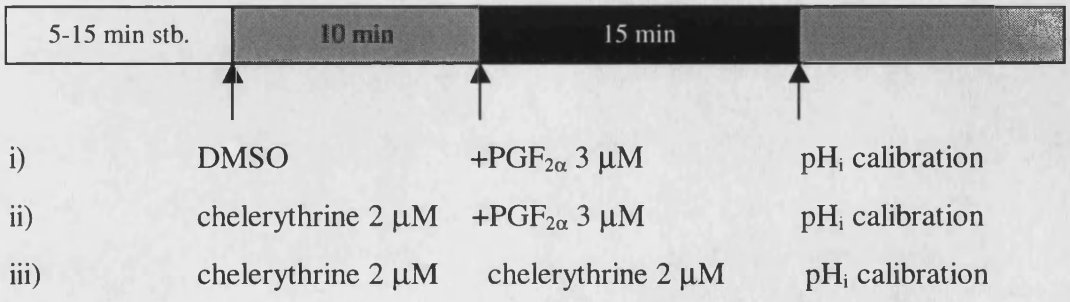
b) Effect of $PGF_{2\alpha}$ on pH_i



c) Effect of the Na^+/H^+ inhibitor, HOE 694, on $PGF_{2\alpha}$-induced increase of pH_i



d) Effect of the PKC inhibitor, chelerythrine, on $PGF_{2\alpha}$-induced increase of pH_i



2.6.4 Phase-plane diagram

A method for assessing changes in the sensitivity of the myofilaments to Ca^{2+} in intact myocytes was described by Spurgeon *et al.* (1992). They argued that during the *late phase of relaxation*, equilibrium conditions between Ca^{2+} binding and contraction exist, which is a prerequisite for the determination of myofilament sensitivity to Ca^{2+}. When myocyte length is plotted with respect to Ca^{2+} during a contraction, a loop or phase-plane diagram results (diagram M-5). Loops proceed in a counter-clockwise direction, and arrows on each trajectory represent 50 ms isochrones. The loop going upwards represents contraction, and the loop going downwards represents the relaxation phase. A shift of the late relaxation phase upwards and leftwards represents an increase in Ca^{2+} sensitivity (diagram M-6), while that downwards and rightwards indicates a reduction in Ca^{2+} sensitivity. This is demonstrated with the Ca^{2+} sensitisers EMD 53998, NH_4Cl-induced alkalosis and phenylephrine, as well with the Ca^{2+} desensitisers butanedione monoxime and isoprenaline (Spurgeon *et al.*, 1992; Gambassi *et al.*, 1992).

Diagram M-5. *A*, simultaneously measured $[Ca^{2+}]_i$ and cell length following stimulation from rest of a representative single rat myocyte that had been loaded with the free acid form of Indo-1. *B*, the initial transients in *A* are depicted with greater resolution. *C*, phase-plane trajectories of cell length-$[Ca^{2+}]_i$ for the transients in *A* (loops proceed in a counter-clockwise direction). Although the individual contractions and $[Ca^{2+}]$ transients vary in magnitude, a common trajectory occurs during relaxation. Modified from Spurgeon *et al.* (1992).

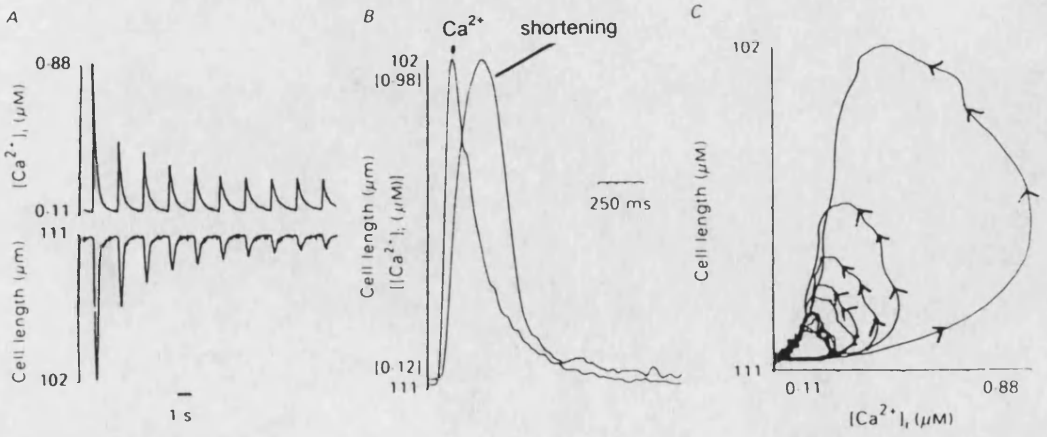
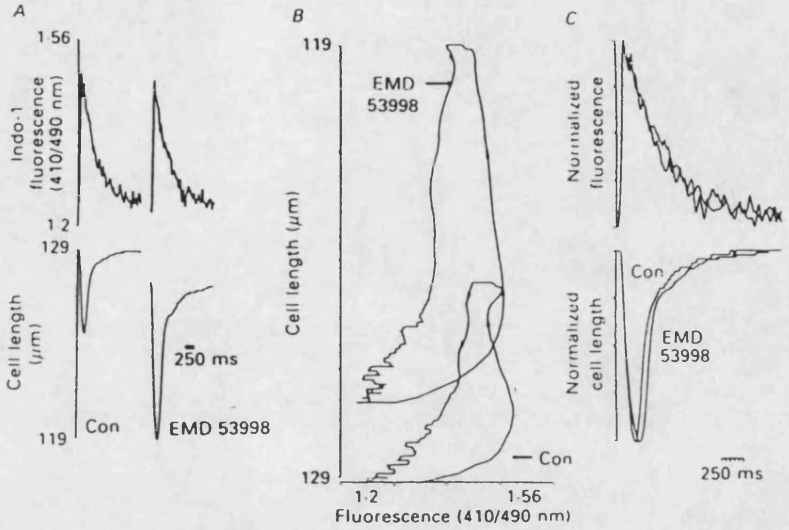


Diagram M-6. Simultaneously measured Indo-1 fluorescence transients and contractions in a representative rat myocyte stimulated at 0.5 Hz prior to and following addition of the Ca^{2+} sensitising drug, EMD 53998, to the bath. *B*, cell length-fluorescence phase-plane trajectories for transients in *A*. *C*, autoscaled fluorescence transients and twitches for events in *A*. Taken from Spurgeon *et al.* (1992).



2.6.4.1 Plotting of the phase-plane diagram

An accurate assessment by this method requires that myocyte length and Ca^{2+} be measured simultaneously, which I was unable to do with the equipment available. Nevertheless, data of myocyte shortening and Ca^{2+} transients already obtained from previous separate experiments (see 2.6.2.4b and 2.6.3.5b) were used to construct a phase-plane diagram to investigate if $PGF_{2\alpha}$ sensitises myofilaments to Ca^{2+}.

The twitch and Ca^{2+} were synchronised so that the difference in time to peak Ca^{2+} and shortening was 100 ms. This time lag period was selected because fura-2 ratios were obtained at 50 ms intervals, and because the lag period approximates to that found previously (Spurgeon *et al.*, 1992).

2.7 Statistical analyses

Values are expressed as means ± standard error of means. They are considered significant when the probability, $P < 0.05$. Where variances were equal, statistical analysis was carried out using one-way ANOVA for differences between groups, and two-way ANOVA for differences within groups. Significance from the control group was determined with Dunnett's method. Paired comparisons within groups were carried out with the one sample Student's *t*-test.

When variances were unequal ($P < 0.05$, Bartlett's test of equal variances), associated non-parametric methods were utilised. The Mann-Whitney U test with Bonferroni correction, after the Kruskal-Wallis ANOVA method, was used for multiple comparisons between groups. In cases where paired data were compared, the Wilcoxon signed rank test was used. Data were tabulated by computer using the statistical analyses package, Minitab for Windows (1994), State College PA.

Results from experiments with single myocytes were usually obtained from one to three myoctes per heart.

SECTION 3

Results

3.1 Isolated perfused rat hearts

This modified Langendorff whole heart model was used to study the effects of $PGF_{2\alpha}$ and the FP receptor agonist, cloprostenol, under constant flow conditions.

a) Effects of $PGF_{2\alpha}$ and cloprostenol on contractility, perfusion pressure and heart rate

$PGF_{2\alpha}$ increased the contractility of the heart in a biphasic manner at doses between 0.01 to 300 nmoles (figures 1 & 3A). Figure 1 shows a small transient increase in contractility which is more prominent at higher doses, preceding a prolonged response to $PGF_{2\alpha}$. A transient decrease in contractility occurred before the increases in contractility. The ED_{50} value was not determined as a plateau was not achieved, although two hearts out of six did show a maximum response at 10 nmoles.

Similarly, the inotropic effect of cloprostenol between 0.01 to 1 nmole was biphasic (figure 2). However, cloprostenol was more potent than $PGF_{2\alpha}$ as the cloprostenol dose response curve shown in figure 3A is to the left of the $PGF_{2\alpha}$ dose response curve. For example, cloprostenol increased developed tension by 26.7 ± 4.1 % (n = 8) at 0.1 nmoles while $PGF_{2\alpha}$ increased developed tension by only 7.7 ± 3 % (n = 6) at the same dose ($P < 0.05$, one-way ANOVA). Also, the response to cloprostenol lasted longer than that of $PGF_{2\alpha}$.

There was a transient vasoconstrictor effect prior to vasodilation in response to $PGF_{2\alpha}$ (figures 1 & 3). In contrast, only a clear vasodilator action was apparent with cloprostenol within the dose range tested (figures 2 & 3).

Figures 1 and 2 show that there was no consistent change in heart rate with either drug.

Figure 1. Typical trace showing the effects of $PGF_{2\alpha}$ on developed tension (DT), heart rate (HR) and coronary perfusion pressure (PP) in isolated rat hearts. One of six similar experiments.

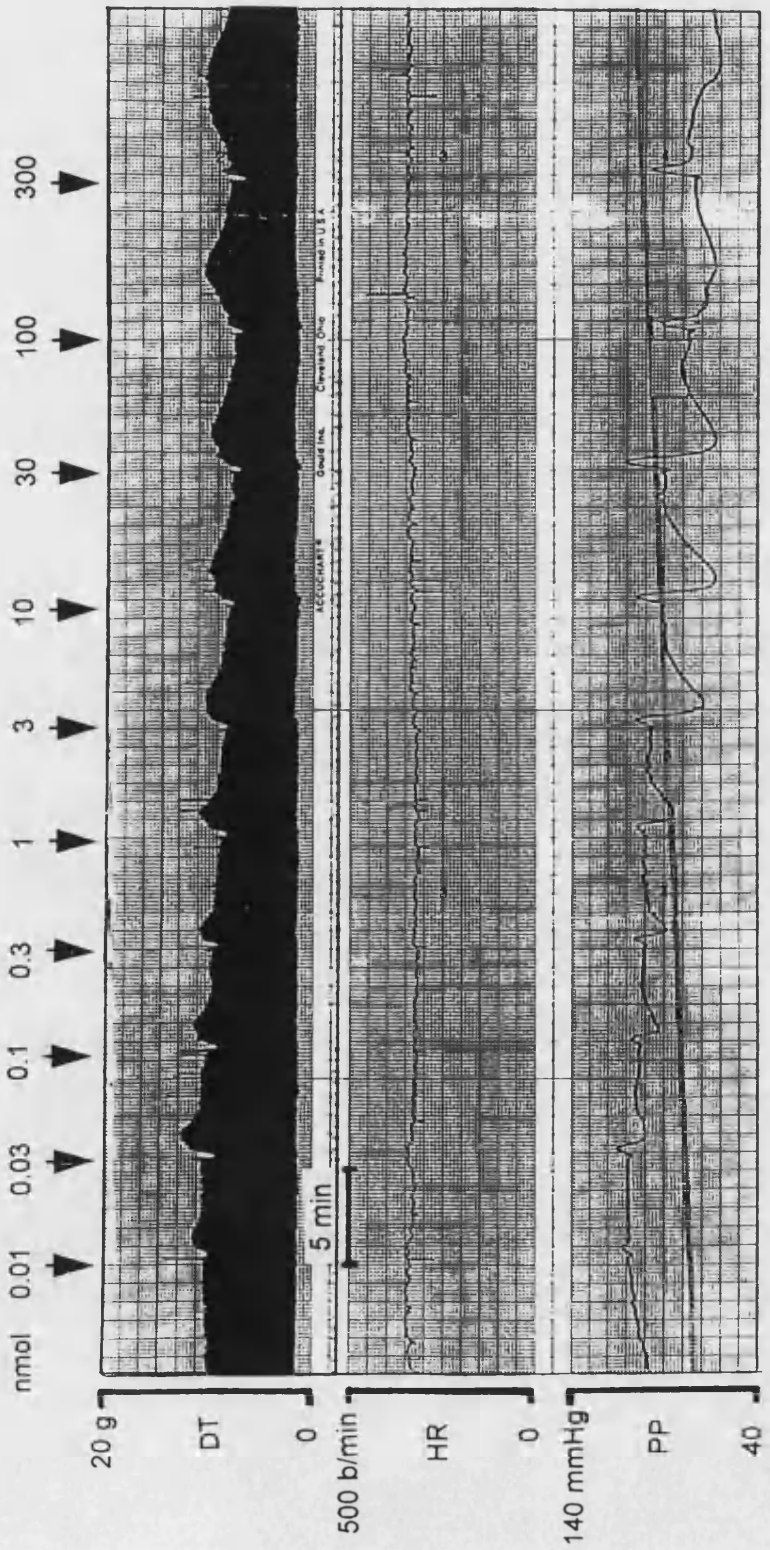


Figure 2. Typical trace showing the effects of cloprostenol on developed tension (DT), heart rate (HR) and coronary perfusion pressure (PP) in isolated rat hearts. One of eight similar experiments.

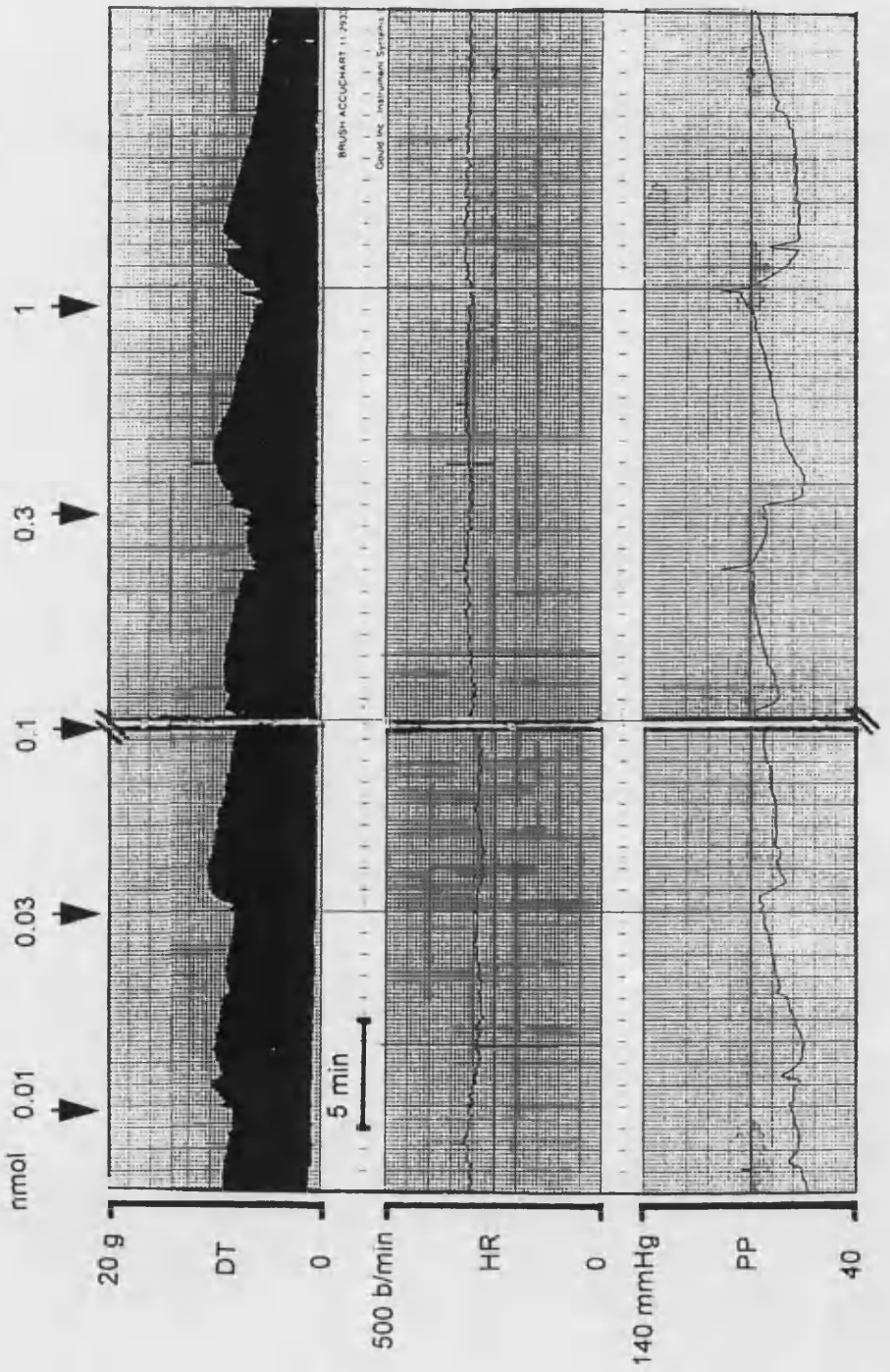
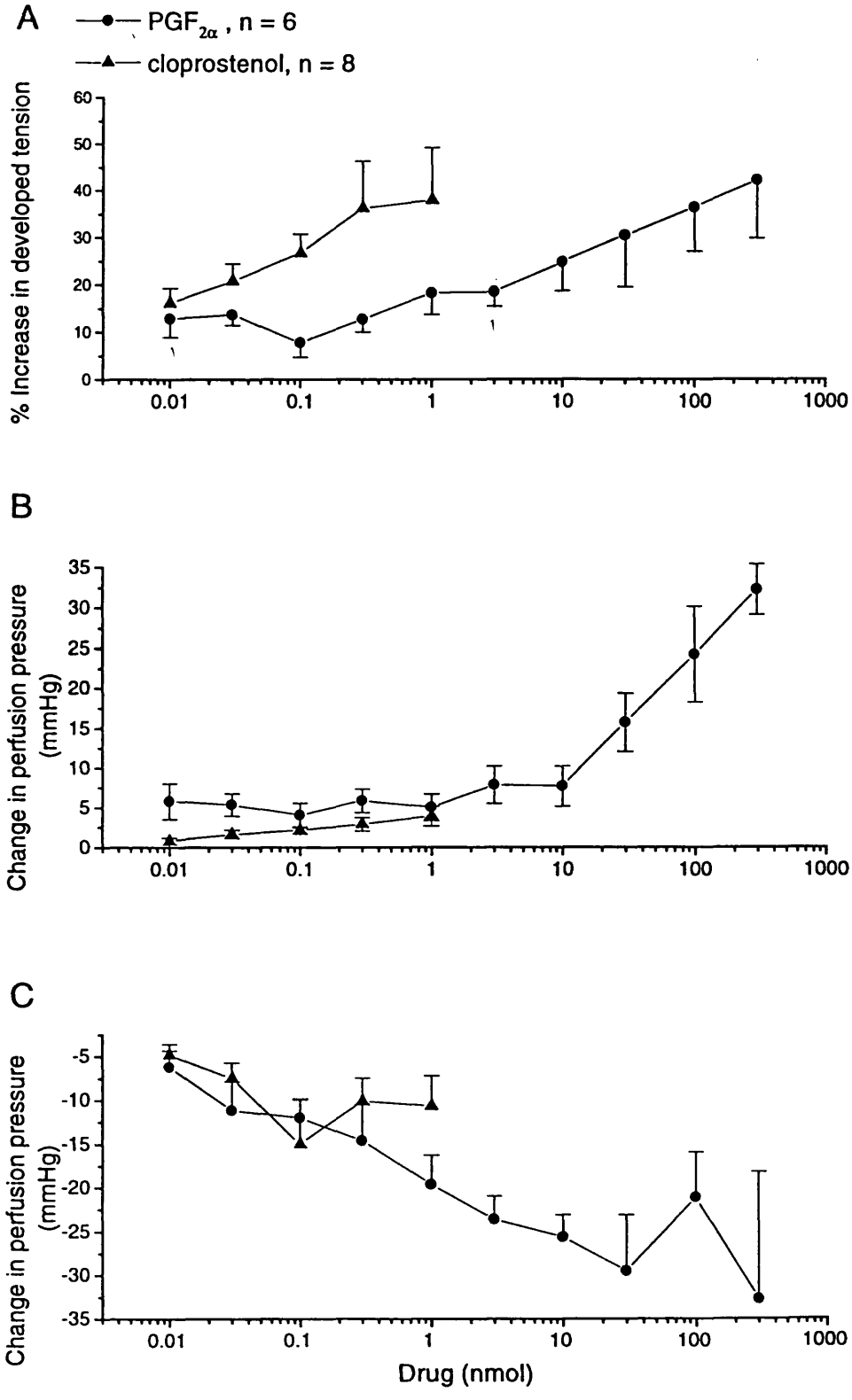


Figure 3. Dose response relationships of $PGF_{2\alpha}$ and cloprostenol on (A) developed tension, (B) initial vasoconstriction and (C) vasodilation in isolated rat hearts. Basal developed tension, heart rate and perfusion pressure was 9.6 ± 0.7 g, 338 ± 17 beats/min and 90 ± 10 mmHg respectively with $PGF_{2\alpha}$, and 8.0 ± 0.4 g, 289 ± 8 beats/min and 75 ± 5 mmHg respectively with cloprostenol.



3.2 Isolated rat left atria

Since $PGF_{2\alpha}$ increased and decreased coronary perfusion pressure in the whole heart, these effects may have influenced the changes in contractility. In order to study contractility in the absence of perfusion pressure changes, electrically stimulated rat left atria were used.

a) Effects of $PGF_{2\alpha}$ and noradrenaline on developed tension

$PGF_{2\alpha}$ increased developed tension of the electrically stimulated left rat atria in a concentration dependent manner, with maximal effects occurring at 1 μM (figures 4 & 5). Figure 5 shows that it was more potent than noradrenaline, as there is a clear shift between their responses. Effects of further concentrations of noradrenaline above 10 μM were not recorded because of an increase in ectopic activity in four out of six atria. In three out of these six experiments, noradrenaline was added in the presence of 10 μM $PGF_{2\alpha}$, a concentration which produced a supramaximal response. This resulted in a further increase in developed tension (figure 6), showing that at least part of their mechanisms of action were different.

b) Effect of $PGF_{2\alpha}$ on the duration of the contraction-relaxation cycle

Figure 7 shows the superimposed effects of different concentrations of $PGF_{2\alpha}$ on the atrial contractile response at a faster chart speed. Time to peak contraction was not altered but relaxation was prolonged by $PGF_{2\alpha}$. This negative lusitropic effect of $PGF_{2\alpha}$ is quantified in figure 8. A proportionally smaller increase in the maximal velocity of relaxation (-T) compared to the maximal velocity of contraction (+T) occurred with 10 μM $PGF_{2\alpha}$, that is, the ratio +T/-T was significantly increased.

Figure 4. Typical trace showing the effects of $PGF_{2\alpha}$ and noradrenaline on developed tension (DT) of the same rat left atria. One of six similar experiments. Note the changes in DT scale when noradrenaline was added.

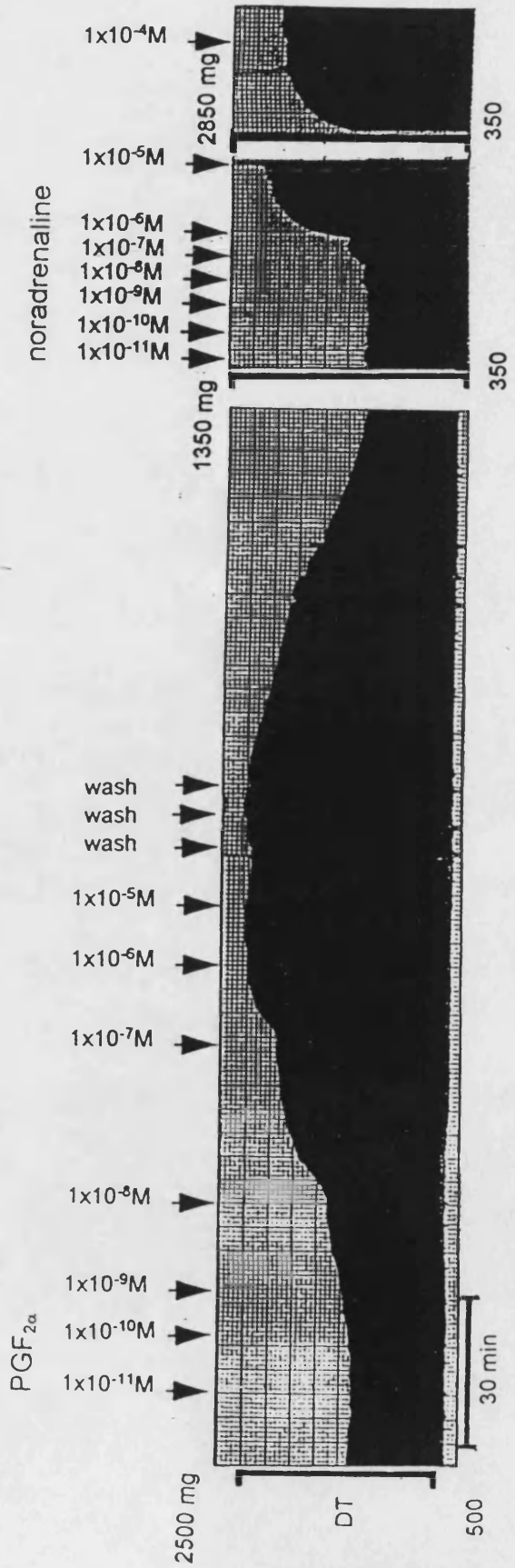


Figure 5. Concentration dependent relationships of $PGF_{2\alpha}$, its vehicle control, and noradrenaline on the contractility of the same rat left atria.

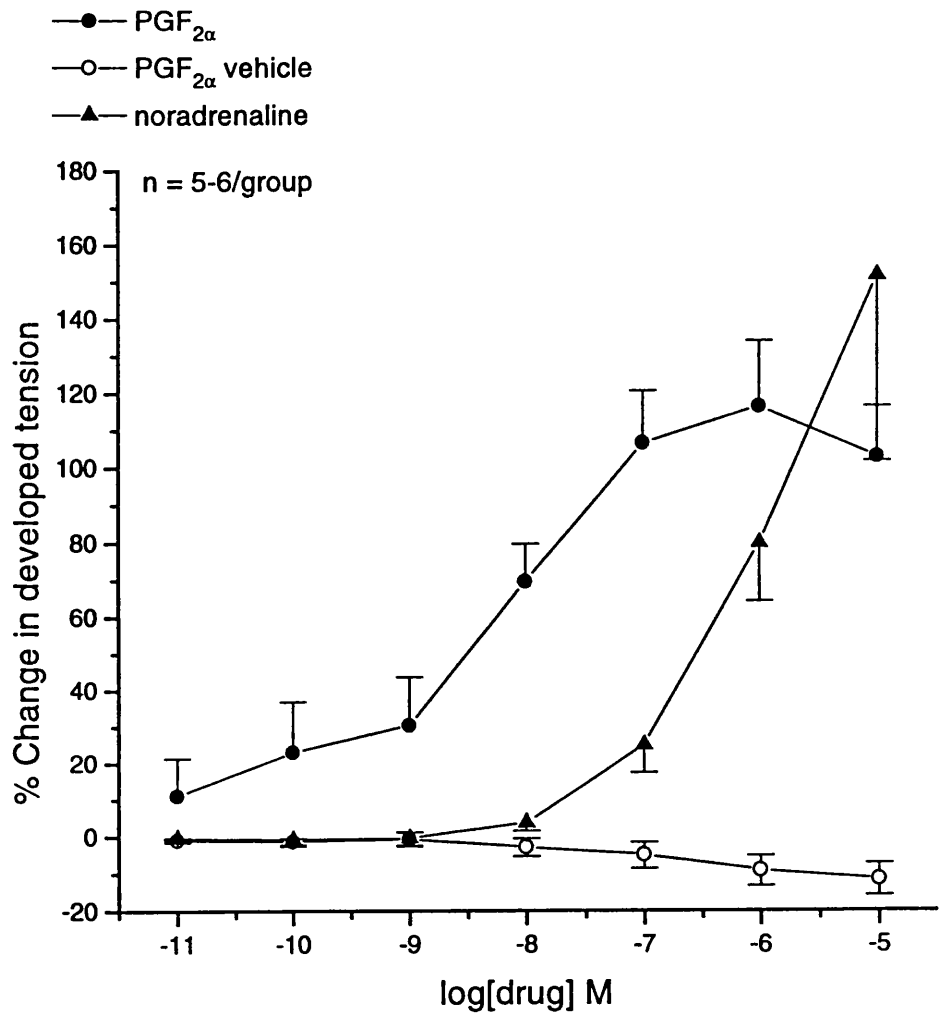


Figure 6. Histogram of the effect of noradrenaline (NAd) on the maximum increase in contractility induced by $PGF_{2\alpha}$ in rat left atria

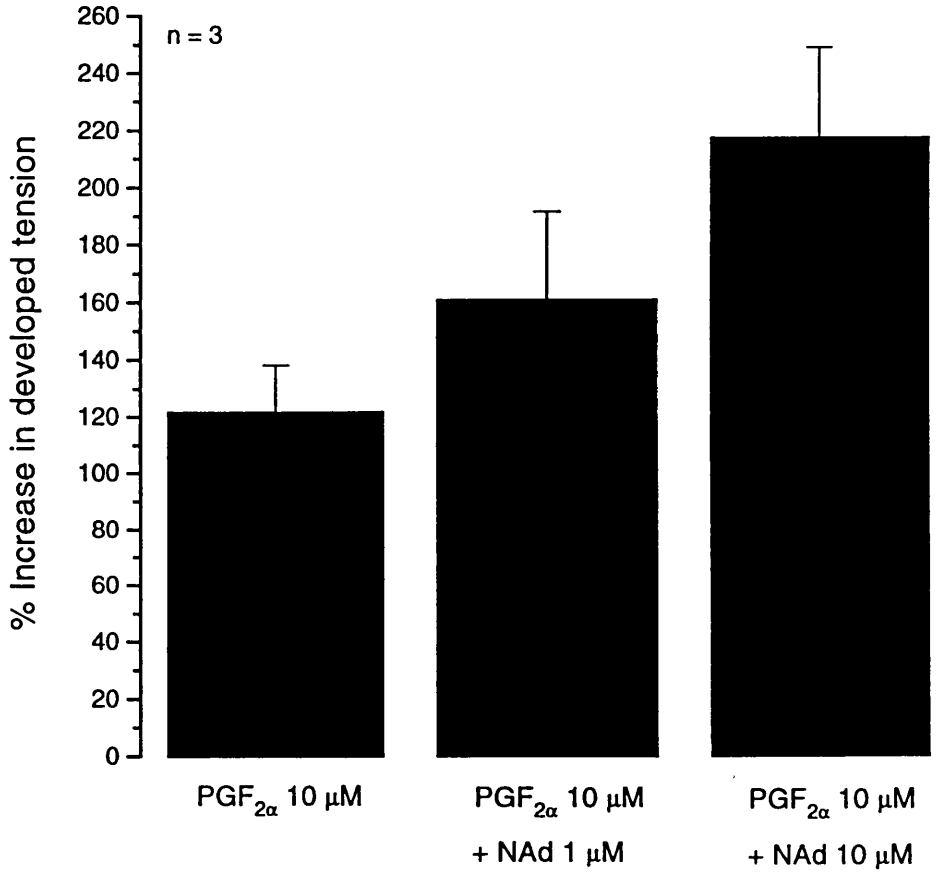


Figure 7. Superimposed traces of the effects of $PGF_{2\alpha}$ on the contraction-relaxation cycle of rat left atrium.

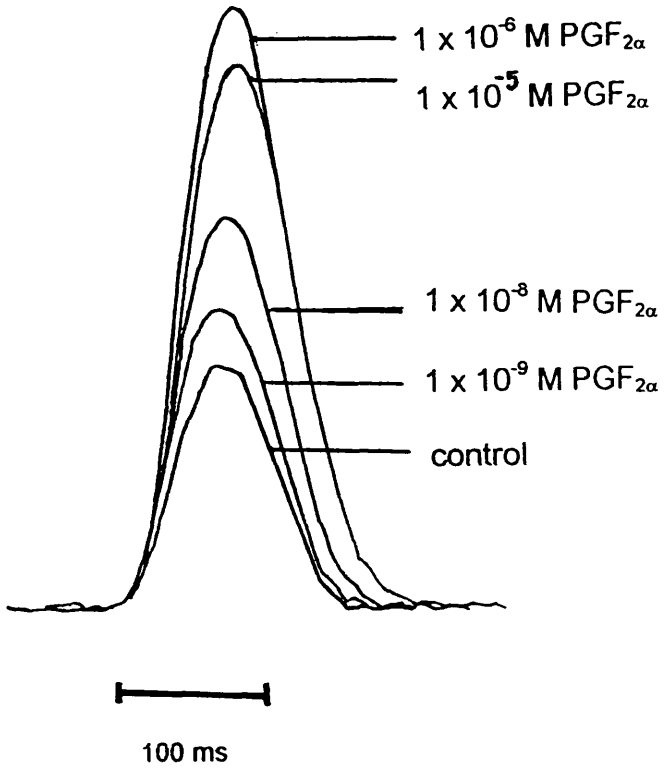
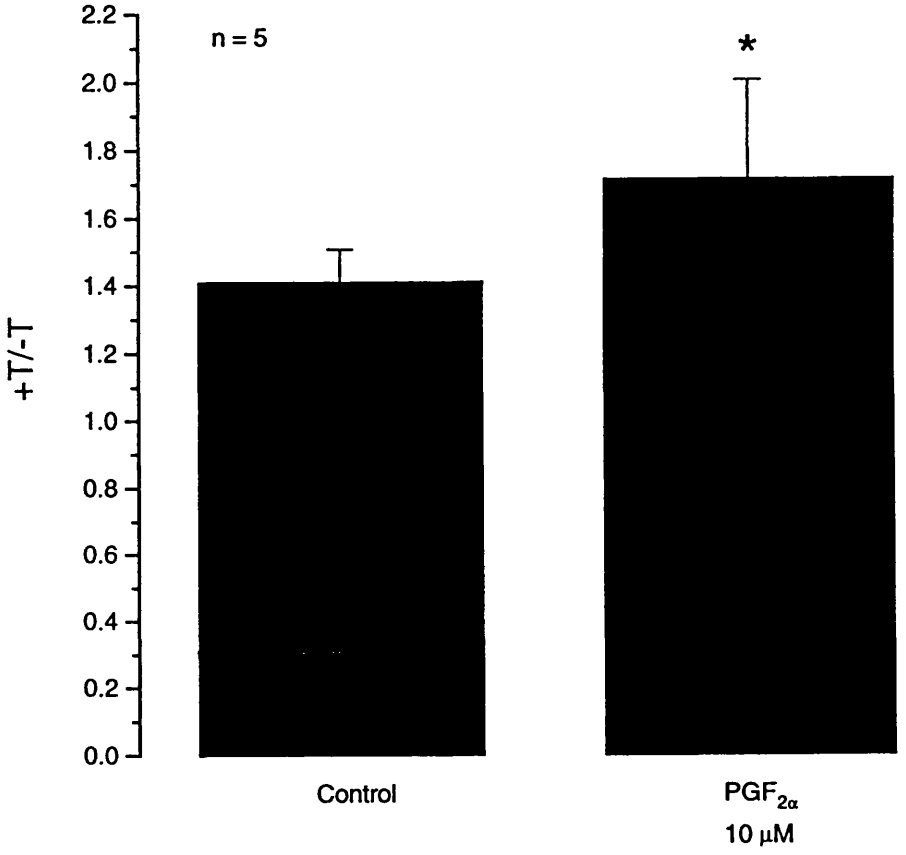


Figure 8. Histogram of the effects of $PGF_{2\alpha}$ on the ratio of the maximum rate of contraction (+T) and the maximum rate of relaxation (-T) of rat left atria. * $p < 0.05$, Wilcoxon signed rank test.



3.3 Isolated rat ventricular myocytes

The mechanism of action of $PGF_{2\alpha}$ was studied at the cellular level using dissociated rat ventricular myocytes, which are devoid of neural and humoral influences. Edge detection was used to study myocyte shortening, while fluorimetric probes fura-2 and BCECF were used to monitor intracellular Ca^{2+} and pH respectively. Figure 9 shows a typical myocyte used in the experiments.

3.3.1 Myocyte shortening experiments

a) Effects of $PGF_{2\alpha}$

Figures 10 and 11 show the concentration dependent increase in myocyte shortening of up to 39.6 ± 3.4% with cumulative addition of $PGF_{2\alpha}$ to 3 μM (n = 4). Additionally, myocyte diastolic length was reduced to 94.3 ± 1.5% of basal length with 3 μM $PGF_{2\alpha}$. Both these effects were reversible upon wash out of the drug. In contrast to results obtained from the atrial preparation (see 3.2b), there was no significant change in the duration of the contraction-relaxation cycle in ventricular myocytes (figures 12 & 13).

b) Effects of fura-2

As fura-2 was to be used to measure intracellular Ca^{2+} (see 3.3.2), some experiments were done to see if fura-2 affected the contractile response to $PGF_{2\alpha}$. Figures 10B & 14 show the increase in myocyte shortening induced by a single concentration of 3 μM $PGF_{2\alpha}$ in fura-2 loaded myocytes. This increase of 40.4 ± 10.7% (n = 7) was similar to that obtained from fura-2 non-loaded myocytes when $PGF_{2\alpha}$ was added cumulatively (39.6 ± 3.4%, n = 4, see 3.3.1a above), indicating that fura-2 was not reducing the increase in myocyte shortening induced by $PGF_{2\alpha}$.

In response to a single concentration of 3 μM, the change in myocyte shortening was either mono- or bi-phasic; three out of seven fura-2 loaded myocytes showed a transient decrease in shortening before the sustained increase in shortening. Similar biphasic effects were seen in myocytes which were not loaded with fura-2 in later experiments (figures 28A and 33A). Not all stably shortening myocytes responded

to $PGF_{2\alpha}$ as one out of seven fura-2 loaded myocytes was unresponsive to 3 μM $PGF_{2\alpha}$. In responding myocytes, the increase in shortening reached a maximum between 5 to 16 minutes. On washout of $PGF_{2\alpha}$, recovery from the increase in shortening was seen in four myocytes. The time course of the contraction-relaxation cycle was unaffected in the presence of $PGF_{2\alpha}$ and fura-2 (figure 15).

c) Relationship between the changes in shortening induced by rapid pacing and that induced by $PGF_{2\alpha}$

In some myocyte batches, the stimulation rate was increased from 1 Hz to 2 Hz and then restored again to 1 Hz to check if the myocyte was likely to last the whole experimental protocol (see 2.6.2.4f). Myocytes that could follow the stimulation train and survive this procedure were used for experiments.

When myocytes were subjected to the pacing protocol described above and followed by the addition of 3 μM $PGF_{2\alpha}$, a significant correlation between the increase/decrease in myocyte shortening due to rapid pacing and that induced by $PGF_{2\alpha}$ was observed, $P = 0.02$ (figure 16). This suggests that there may be a similarity between both the mechanisms. This observation was later used to ensure that myocytes in all groups during an experiment had similar basal responses to $PGF_{2\alpha}$ (see 3.3.5c).

In a separate group of experiments, the same rapid pacing protocol was used to determine the proportion of myocytes that displayed the different contractile effects during rapid pacing (staircase effects). In a total of 50 myocytes from four hearts, 34 myocytes showed a positive staircase (increased shortening), 4 showed a flat staircase (no change), while 12 myocytes showed a negative staircase (decreased shortening).

Figure 9. Microscope image of a single rat ventricular myocyte, magnified 536 times.

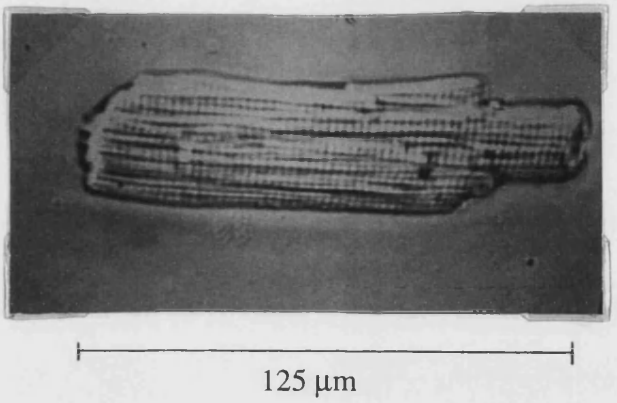


Figure 10. Typical traces showing the effects of $PGF_{2\alpha}$ in rat ventricular myocytes not loaded with fura-2 (A) and that loaded with fura-2 (B). One of four and seven similar experiments respectively.

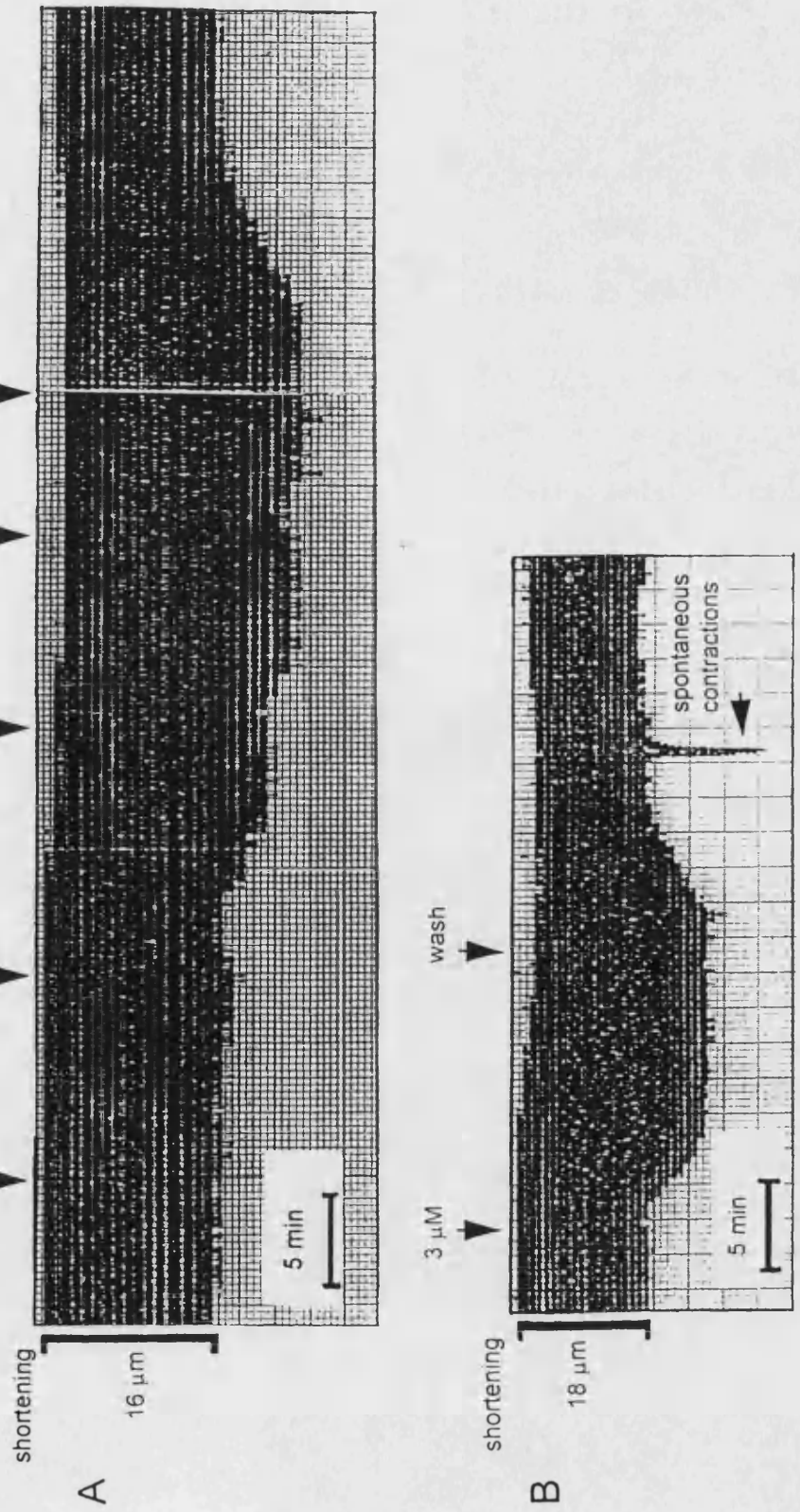


Figure 11. Concentration dependent effects of cumulative addition of $PGF_{2\alpha}$ on shortening (A) and diastolic length (B) of rat ventricular myocytes. Basal shortening was 12.2 ± 1.1% of myocyte length. Basal diastolic length was 128 ± 11 μm.

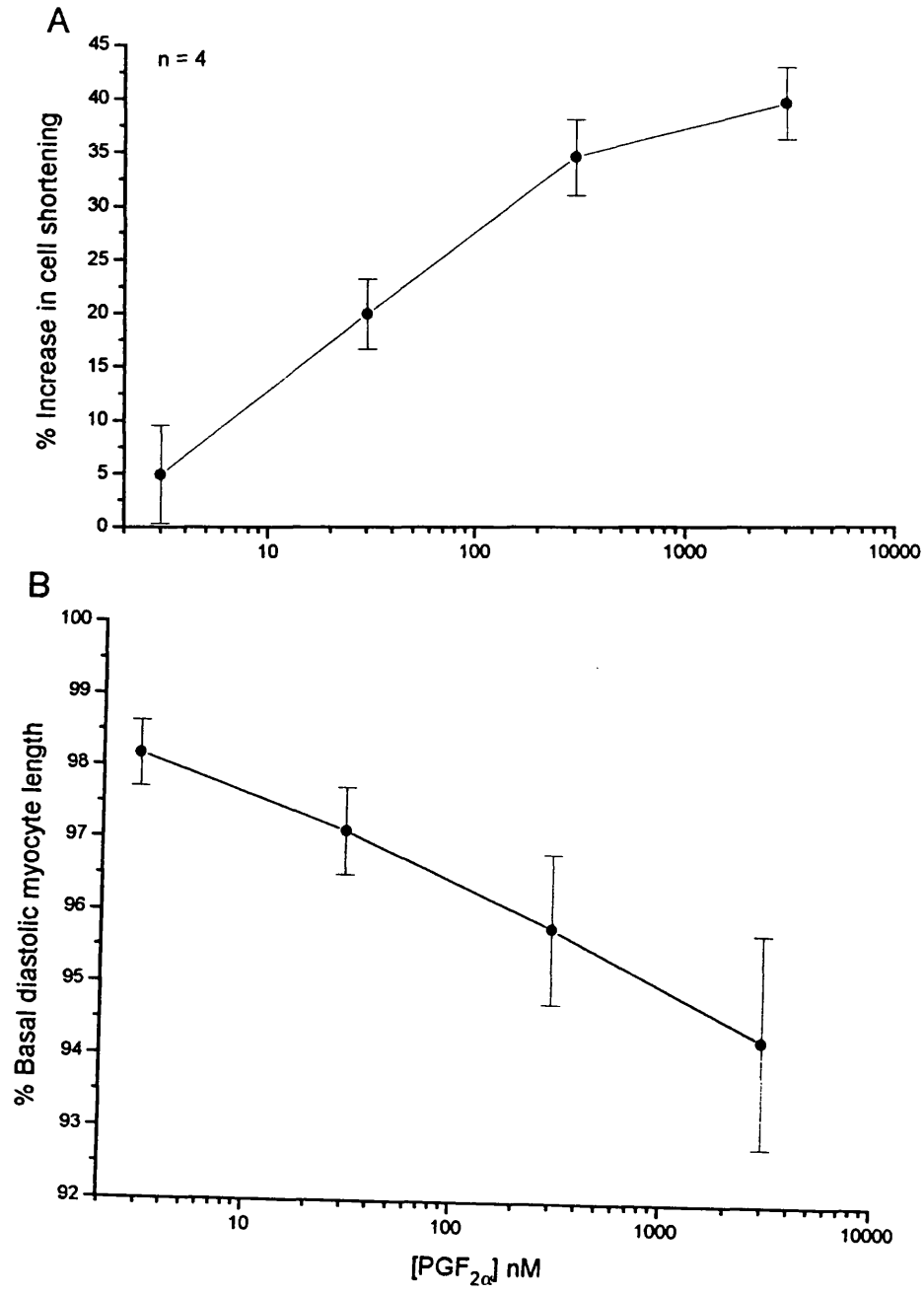


Figure 12. Concentration dependent effects of cumulative addition of $PGF_{2\alpha}$ on the rat ventricular myocyte contraction-relaxation cycle.

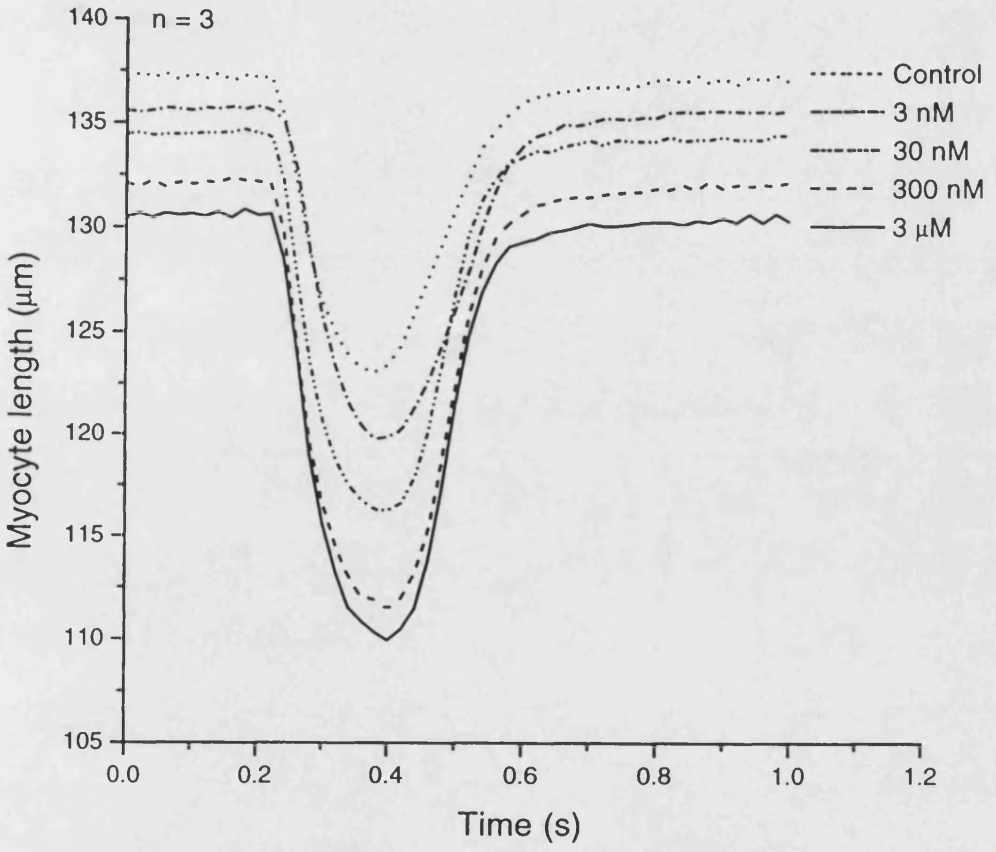


Figure 13. Concentration dependent effects of cumulative addition of $PGF_{2\alpha}$ on rat ventricular myocyte time to peak shortening, TTP (A), time to 50% relaxation, R50 (B) and time to 90% relaxation, R90 (C).

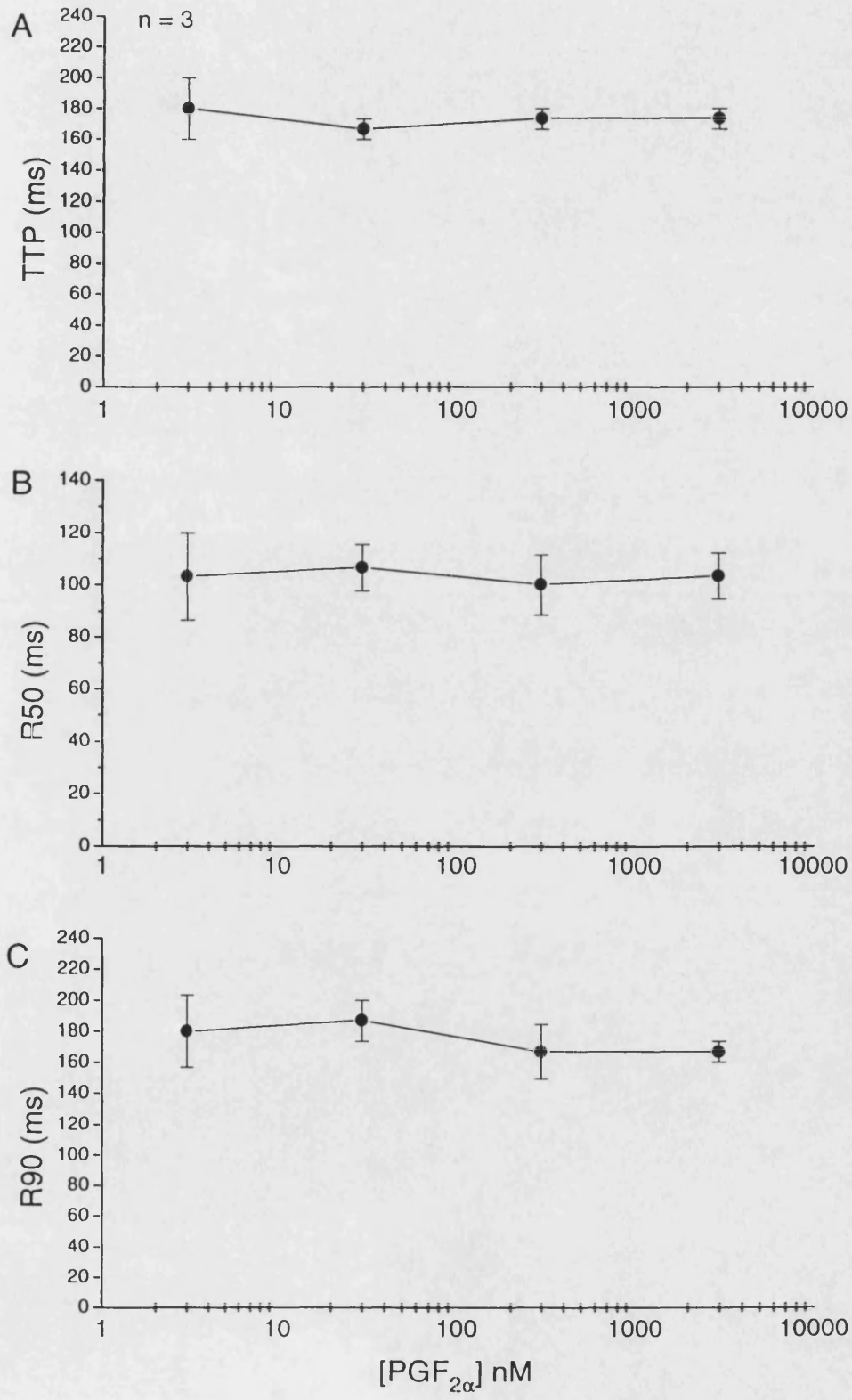


Figure 14. Effect of $PGF_{2\alpha}$ on shortening of fura-2 loaded rat ventricular myocytes.

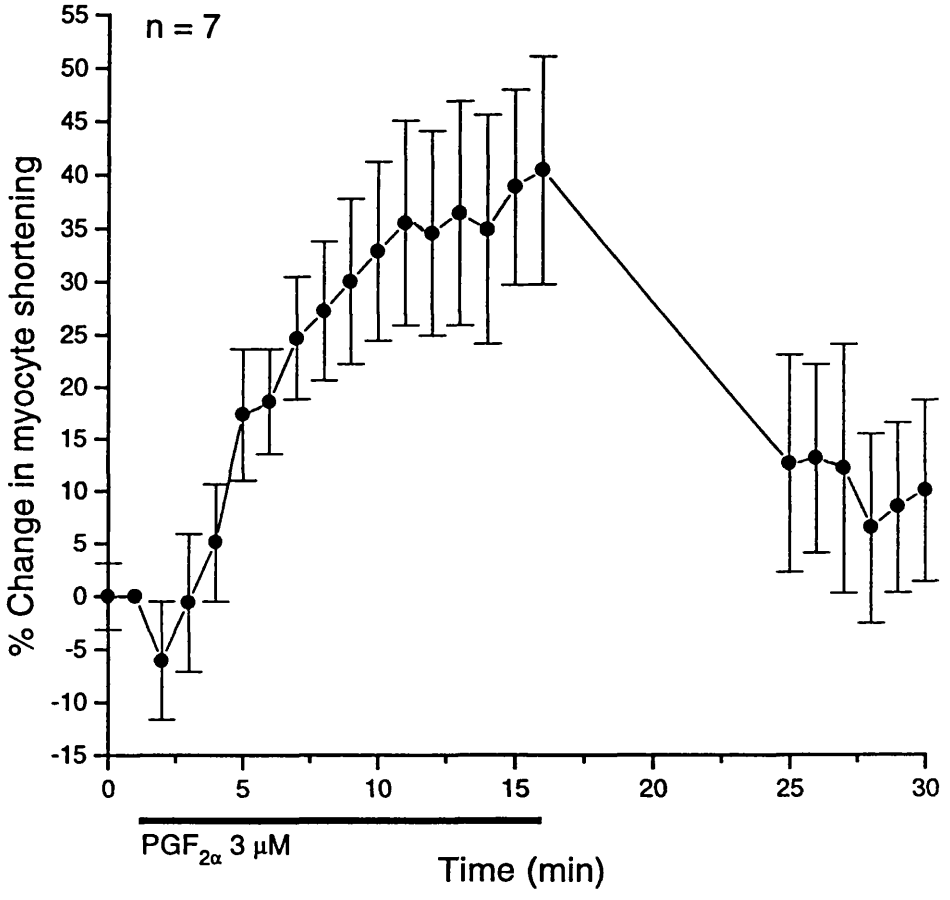


Figure 15. Effects of $PGF_{2\alpha}$ on the fura-2 loaded rat ventricular myocyte time to peak shortening, TTP (A), time to 50% relaxation, R50 (B), and time to 90% relaxation, R90 (C).

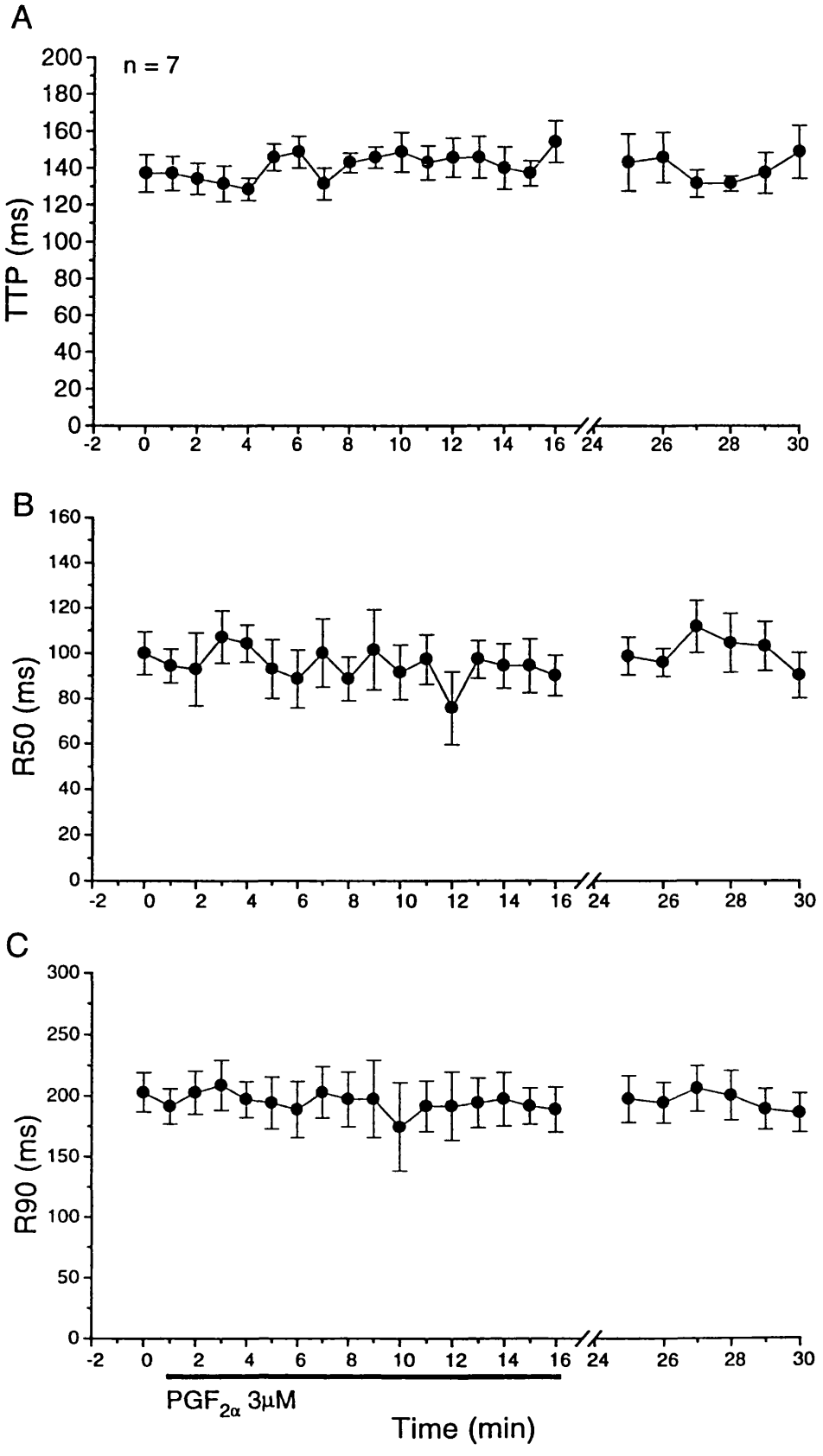
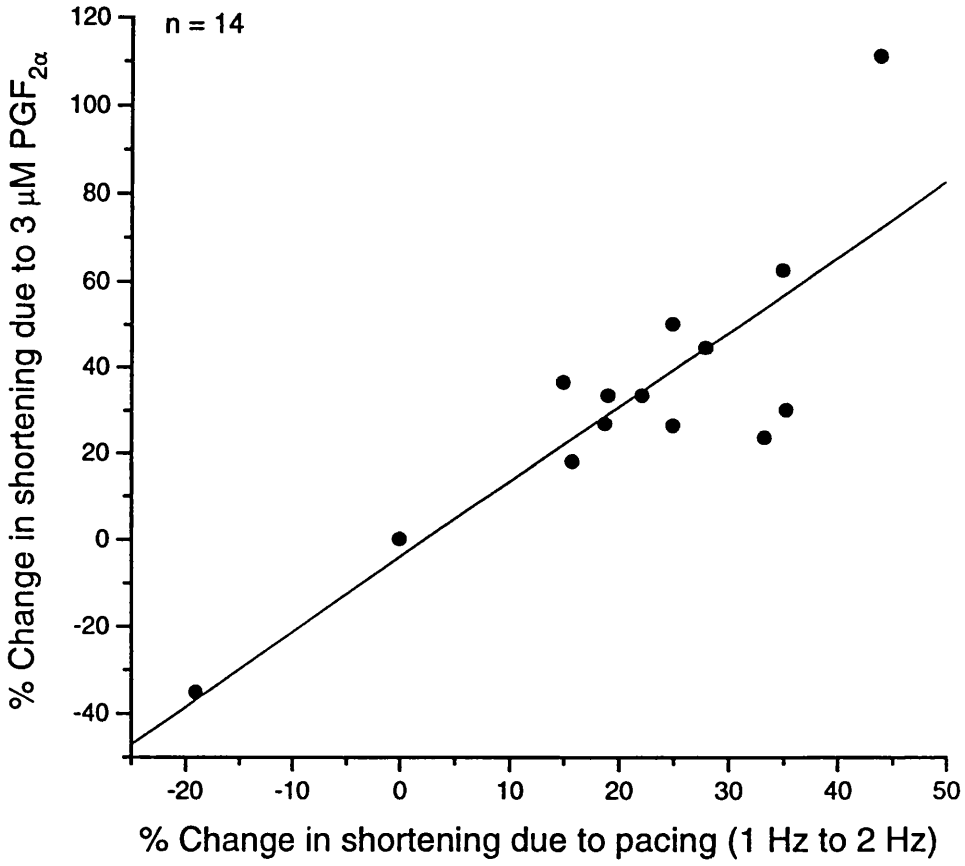


Figure 16. Correlation between the increase in shortening induced by $PGF_{2\alpha}$ and that by increasing pacing frequency in rat ventricular myocytes (refer to protocol 2.6.2.4f). Pearson correlation = 0.623, P= 0.02.



3.3.2 Ca^{2+} fluorescence and Ca^{2+} current experiments

Electrical stimulation of fura-2 loaded myocytes caused a rapid increase and a gradual decline in the fura-2 340/380 fluorescence ratio with each myocyte shortening. Noradrenaline was used in preliminary experiments as a positive control to show that changes in Ca^{2+} transients could be detected before the effects of $PGF_{2\alpha}$ were tested. The amplitude of the Ca^{2+} transients, diastolic Ca^{2+} and duration of the transient decay were recorded. Ca^{2+} currents were also recorded in $PGF_{2\alpha}$-treated myocytes patch-clamped in the whole cell configuration.

a) Effects of noradrenaline on Ca^{2+} transients

Noradrenaline (0.03 & 300 nM) increased the amplitude of the 340/380 fluorescence ratio in fura-2 loaded myocytes, and this is shown in figures 17 and 18. Also, diastolic 340/380 ratio was transiently reduced with 300 nM noradrenaline before a sustained significant increase occurred (figure 18). The duration of the Ca^{2+} transients was attenuated at the point of 5% of the peak amplitude above the diastolic level (figures 17 & 21). Therefore, changes in myocyte intracellular Ca^{2+} levels were detectable spectrofluorimetrically and 300 nM noradrenaline was used as a positive control.

b) Effect of $PGF_{2\alpha}$ on Ca^{2+} transients

$PGF_{2\alpha}$, at a concentration of 3 μM that produced a marked increase in myocyte shortening, did not significantly increase the amplitude or change the diastolic 340/380 fluorescent ratio of nine fura-2 loaded myocytes within seven minutes (figures 17, 19 & 20). Duration of the Ca^{2+} transients was also not significantly changed (figures 17 & 21). In three of these myocytes, washout of $PGF_{2\alpha}$ and superfusion of these same myocytes with 300 nM noradrenaline produced a clear increase in the amplitude of the 340/380 ratio with an initial decrease followed by recovery of the diastolic 340/380 ratio (figure 20). These results indicate that although no change in the Ca^{2+} transients was seen with $PGF_{2\alpha}$, the myocytes were capable of responding to agonists that increase intracellular Ca^{2+} levels.

c) Effect of $PGF_{2\alpha}$ on L-type Ca^{2+} currents

Dr. Katherine Reeves measured L-type Ca^{2+} currents in myocytes patched clamped in the whole cell configuration. $PGF_{2\alpha}$ (100 nM) did not affect the Ca^{2+} current of these myocytes, but addition of isoprenaline (100 nM) at the end of the experiment did increase the Ca^{2+} current (figure 22).

Figure 17. Superimposed traces showing the effects of $PGF_{2\alpha}$ and noradrenaline on Ca^{2+} transients from the same rat ventricular myocyte. One of three similar experiments.

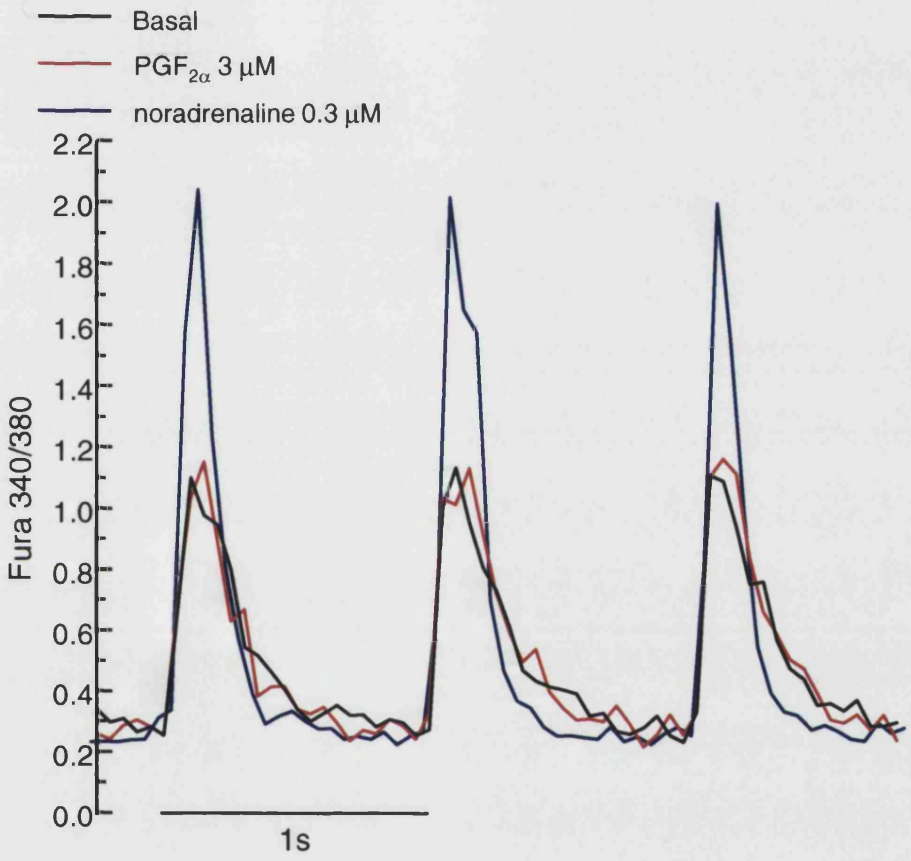


Figure 18. Effects of noradrenaline on the intracellular Ca^{2+} level in rat ventricular myocytes. This is shown by changes in the amplitude (A) and diastolic (B) fura-2 340/380 ratio. ** $P < 0.01$ with respect to time = 50s (two way ANOVA with Dunnett's test for multiple comparisons).

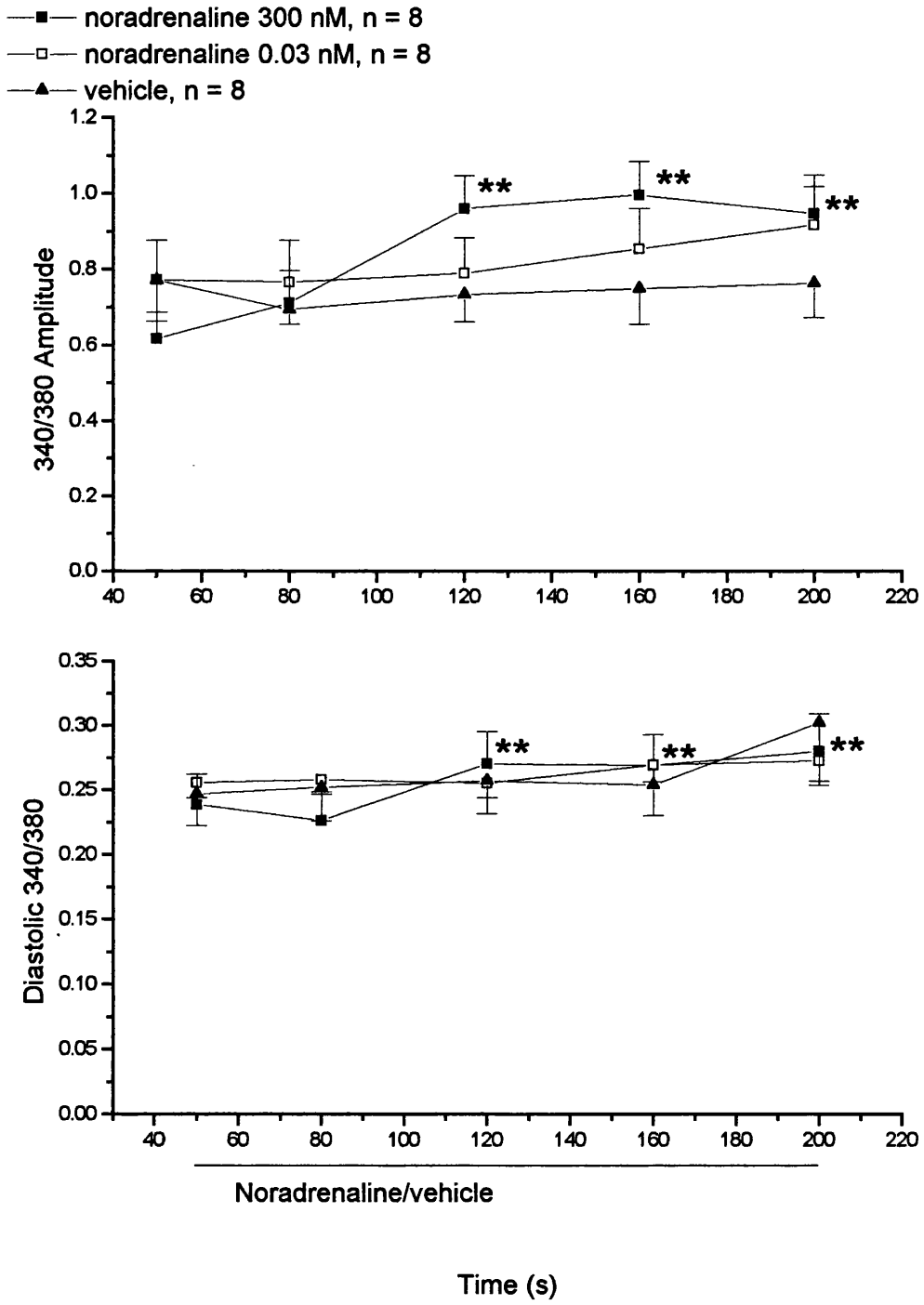


Figure 19. The lack of effect of $PGF_{2\alpha}$ and its vehicle with respect to the amplitude (A) and diastolic (B) fura-2 340/380 ratio of rat ventricular myocytes.

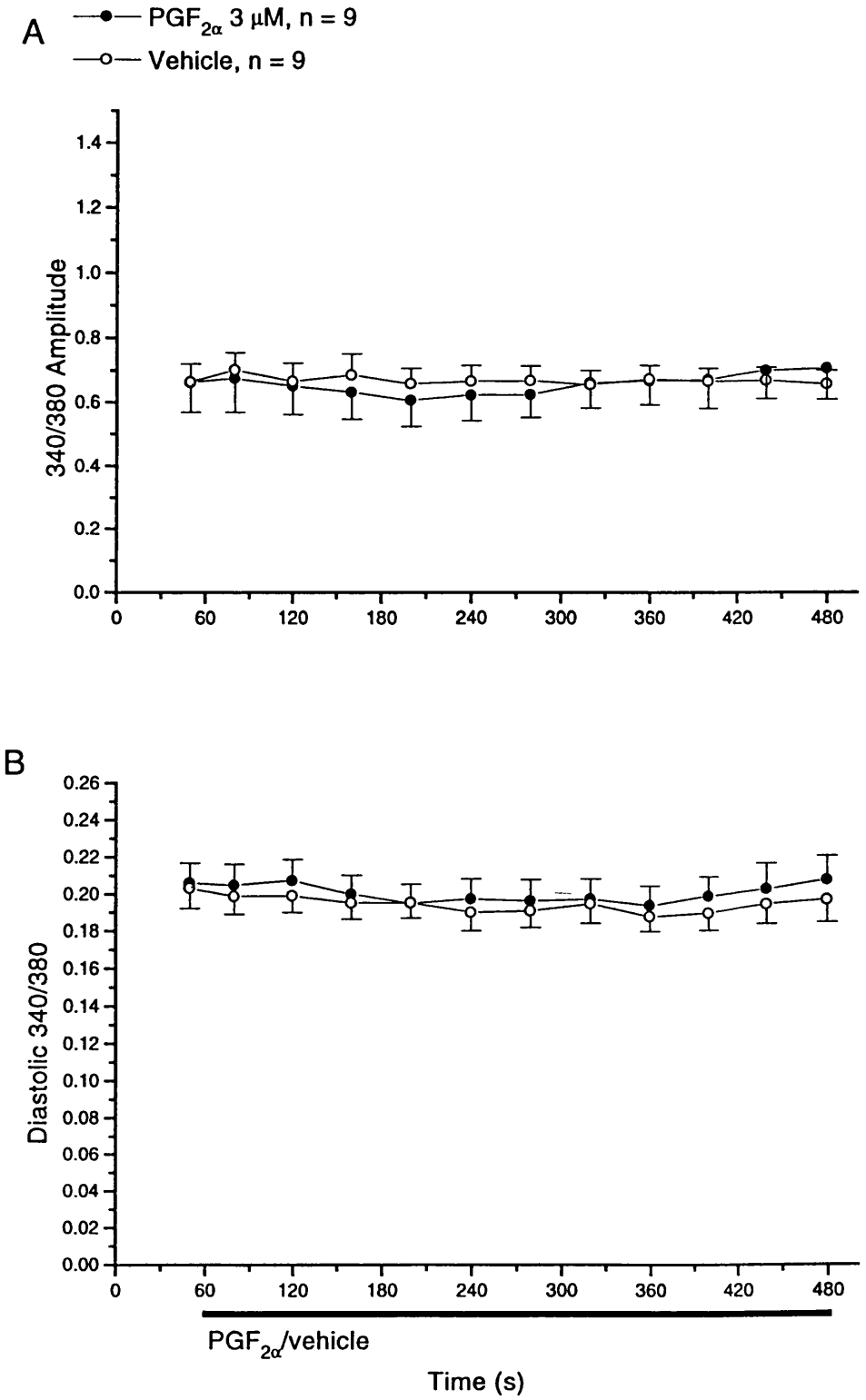


Figure 20. Effects of $PGF_{2\alpha}$ and its vehicle on the amplitude (A) and diastolic (B) fura-2 340/380 ratio of rat ventricular myocytes. In these experiments, noradrenaline was added at the end to show that myocytes could respond to agonists that increase Ca^{2+} transients.

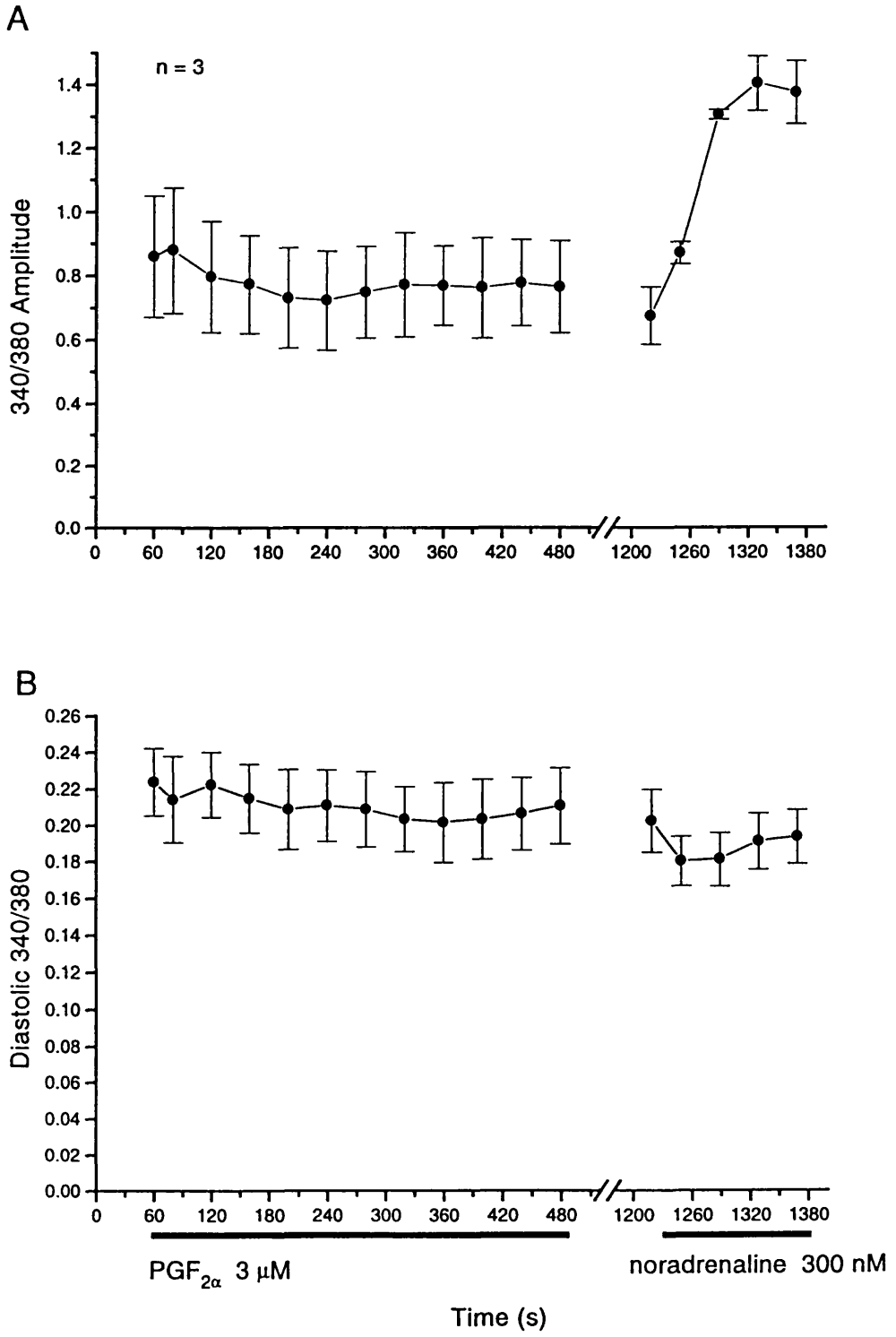


Figure 21. Histogram indicating the change of the duration of the Ca^{2+} transient by noradrenaline but not by $PGF_{2\alpha}$ in rat ventricular myocytes. This was measured as the duration of the fura-2 340/380 ratio spike width at 5% of the amplitude value above basal after 100 s with noradrenaline and its vehicle, or after 7 min with $PGF_{2\alpha}$ and its vehicle. *** $P < 0.001$, paired *t*-test.

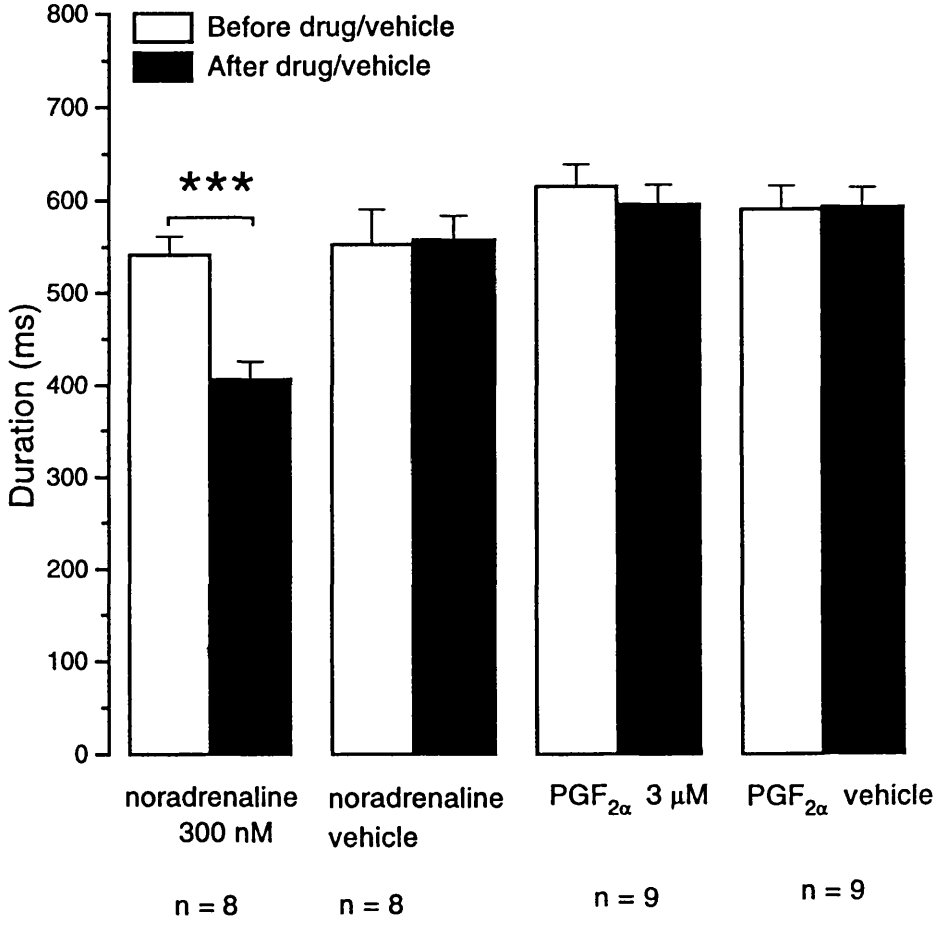
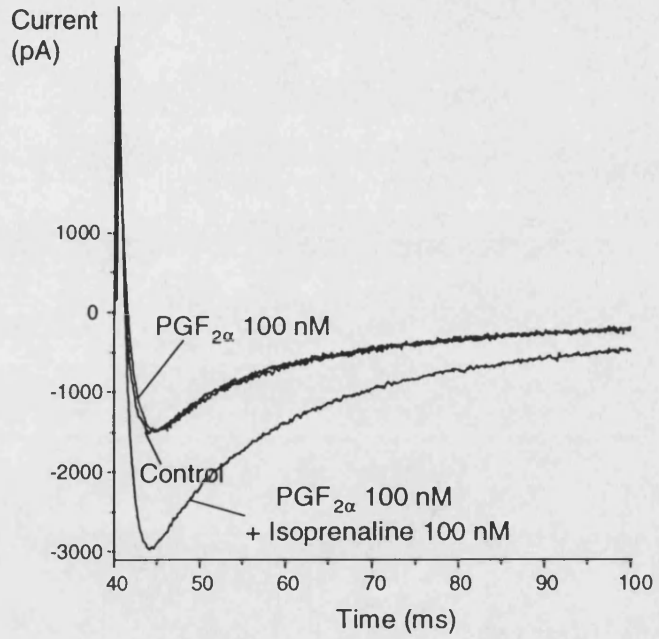


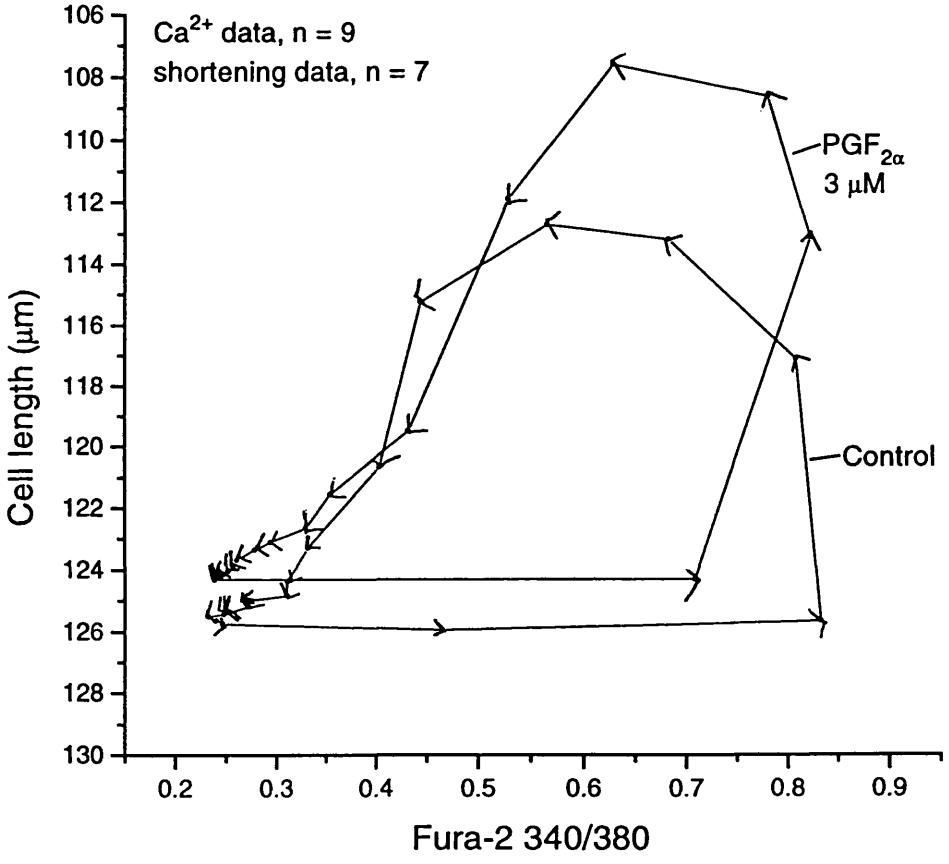
Figure 22. Typical trace of the effects of $PGF_{2\alpha}$ and isoprenaline on the L-type Ca^{2+} currents in the same rat ventricular myocyte. One of three similar experiments where isoprenaline was added at the end of the experiment. Myocytes were patch clamped in the whole cell configuration at 32°C and Ca^{2+} currents elicited by depolarising the cell every 20 s from a holding potential of -80 mV to 0 mV for 200ms. Fast Na^{+} currents were eliminated using a pre-pulse to -40 mV for 40 ms prior to the test pulse. This work was done by Dr. Katherine Reeves.



3.3.3 Effect of $PGF_{2\alpha}$ on the phase-plane diagram

Data from the Ca^{2+} transient and myocyte shortening experiments were plotted together with respect to time, resulting in a phase-plane diagram which gave an indication of the Ca^{2+} sensitivity of the myofilaments. It should be noted that Ca^{2+}/myocyte shortening recordings were not taken from the same cells. However, figure 23 shows that $PGF_{2\alpha}$ (3 μM) did shift the late phase of relaxation upwards and to the left when compared to control.

Figure 23. Effect of $PGF_{2\alpha}$ on the late phase of relaxation of the phase-plane diagram from data obtained from rat ventricular myocytes. Each arrow represents a 50 ms isochrone. Standard error of means are omitted for clarity.



3.3.4 pH_i fluorescence experiments

Previous experiments in fura-2 loaded myocytes showed that $PGF_{2\alpha}$ did not increase Ca^{2+} transients. Therefore, the possibility that $PGF_{2\alpha}$ increased myocyte shortening by increasing intracellular pH arose because some positive inotropic agents do not affect Ca^{2+} transients but do increase intracellular pH. Alkalosis has been reported to alter Ca^{2+} sensitivity of contractile proteins (Fabiato & Fabiato, 1978), so this possibility was investigated in BCECF loaded myocytes treated with $PGF_{2\alpha}$.

a) Calibration of pH_i

The BCECF 490/440 ratio was linearly related to pH between pH 6.8 to 7.6 when calibrated with the nigericin method. This is shown in figure 24, where $R = 0.99$, $p = 0.005$.

b) Effect of $PGF_{2\alpha}$ on pH_i

Figures 25 and 26 show that 3 μM $PGF_{2\alpha}$ increased pH_i of BCECF loaded myocytes by 0.08 ± 0.01 pH units. This effect was reversible upon wash out. This intracellular alkalosis occurred at a similar time course to that of the increase in myocyte shortening seen in the fura-2 loaded myocytes in separate experiments (figure 27). In contrast, time matched vehicle controls showed a gradual decline in myocyte pH_i of about 0.003 units/min (figure 26).

Figure 24. (A) Typical calibration trace of myocyte pH_i. One of 34 similar experiments. (B) Change of the 490/440 BCECF ratio with respect to pH by the nigericin method in rat ventricular myocytes. R = 0.99, P= 0.005.

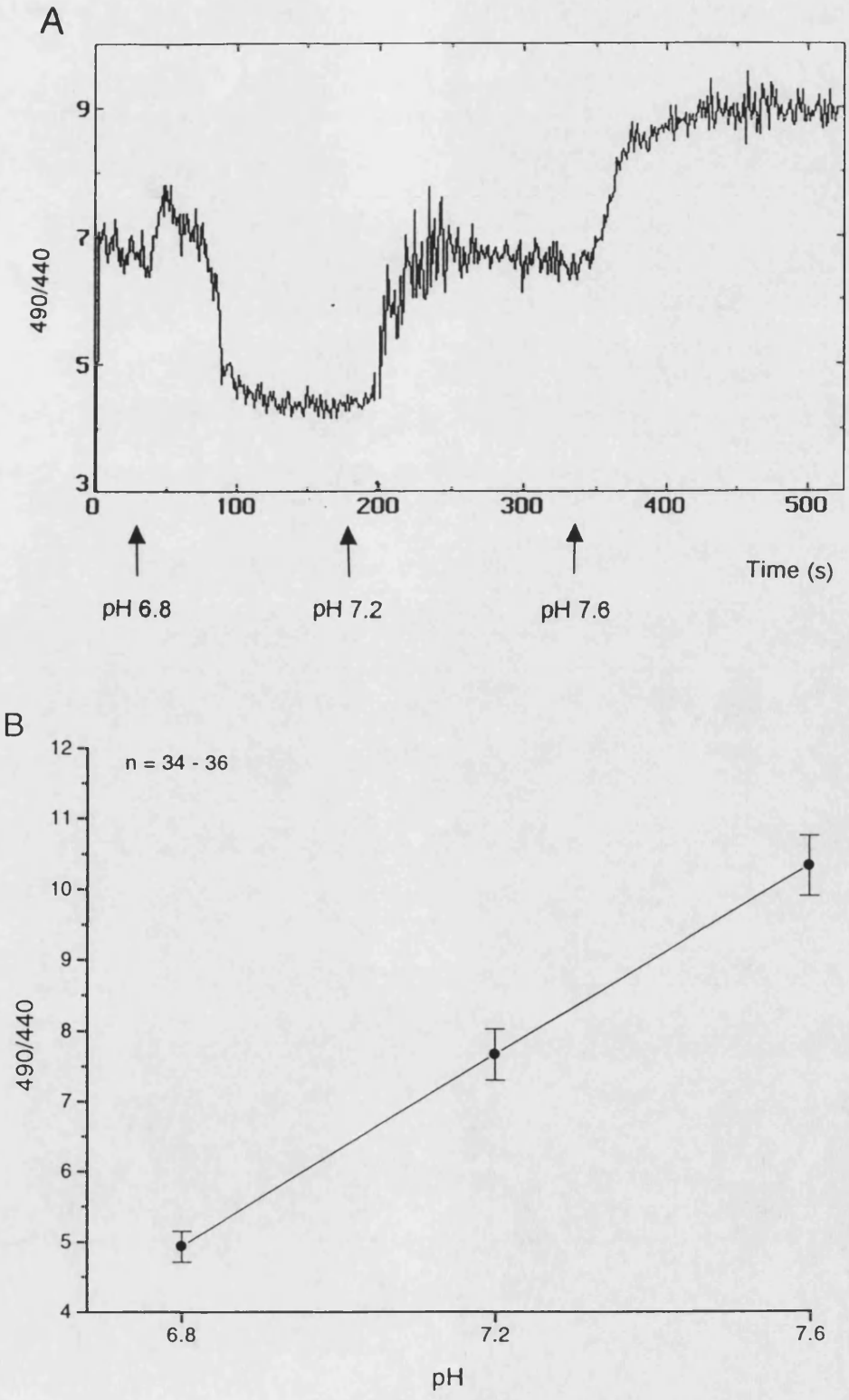


Figure 25. Typical trace of the effect of $PGF_{2\alpha}$ on pH_i of BCECF loaded rat ventricular myocytes, one of five similar experiments. Measurements were taken at 1 min intervals for a 10 s duration during a 40 min period. Inserts (A) and (B) show the amplified timescale at the point of, and 15 minutes after, $PGF_{2\alpha}$ administration.

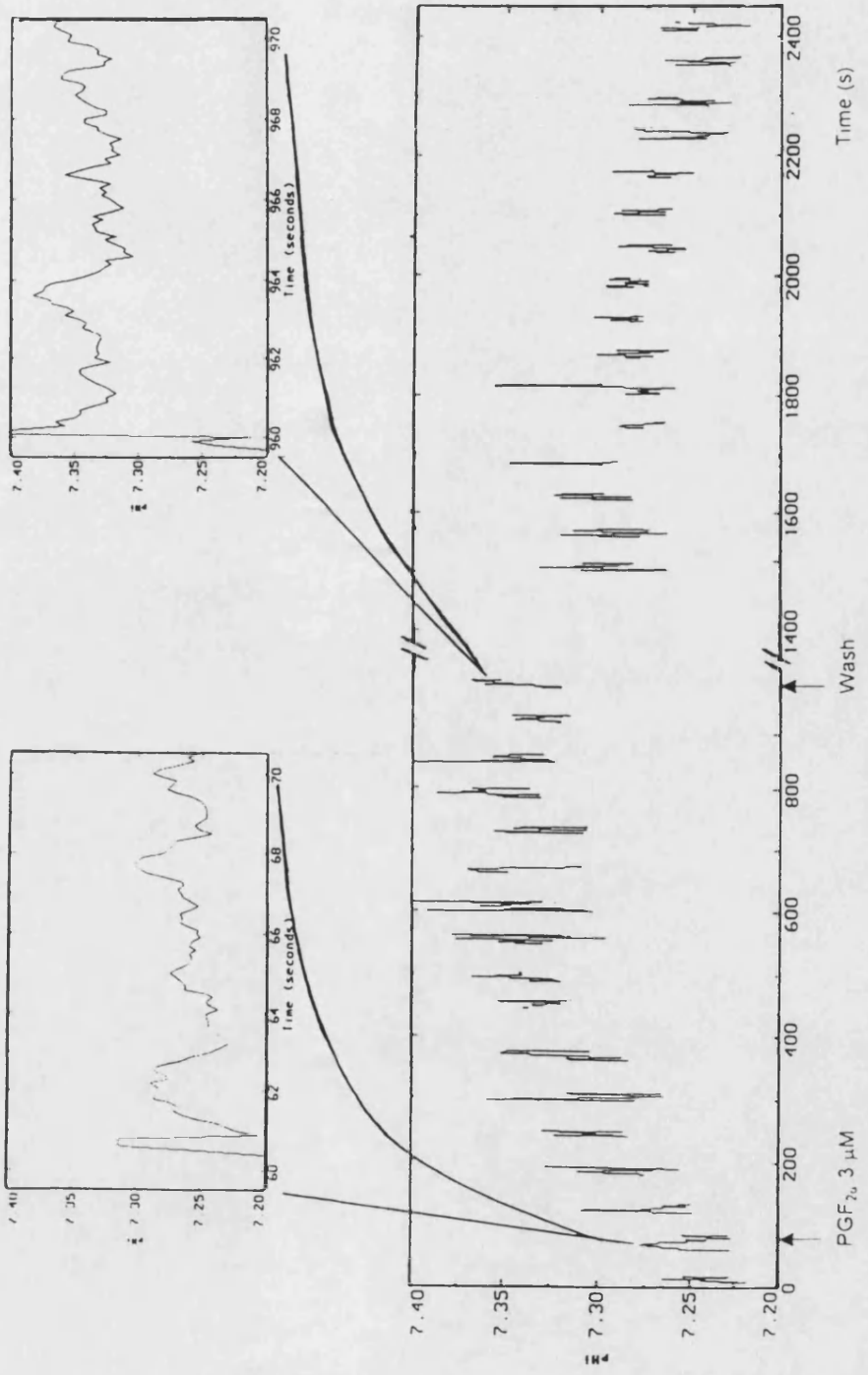


Figure 26. Effect of $PGF_{2\alpha}$ on pH_i of BCECF loaded rat ventricular myocytes. Wash out of $PGF_{2\alpha}$ after t = 16 min reversed its effect on pH_i. pH_i was standardised at the start of $PGF_{2\alpha}$ addition. * $P < 0.05$ compared to its corresponding vehicle time matched controls, one-way ANOVA.

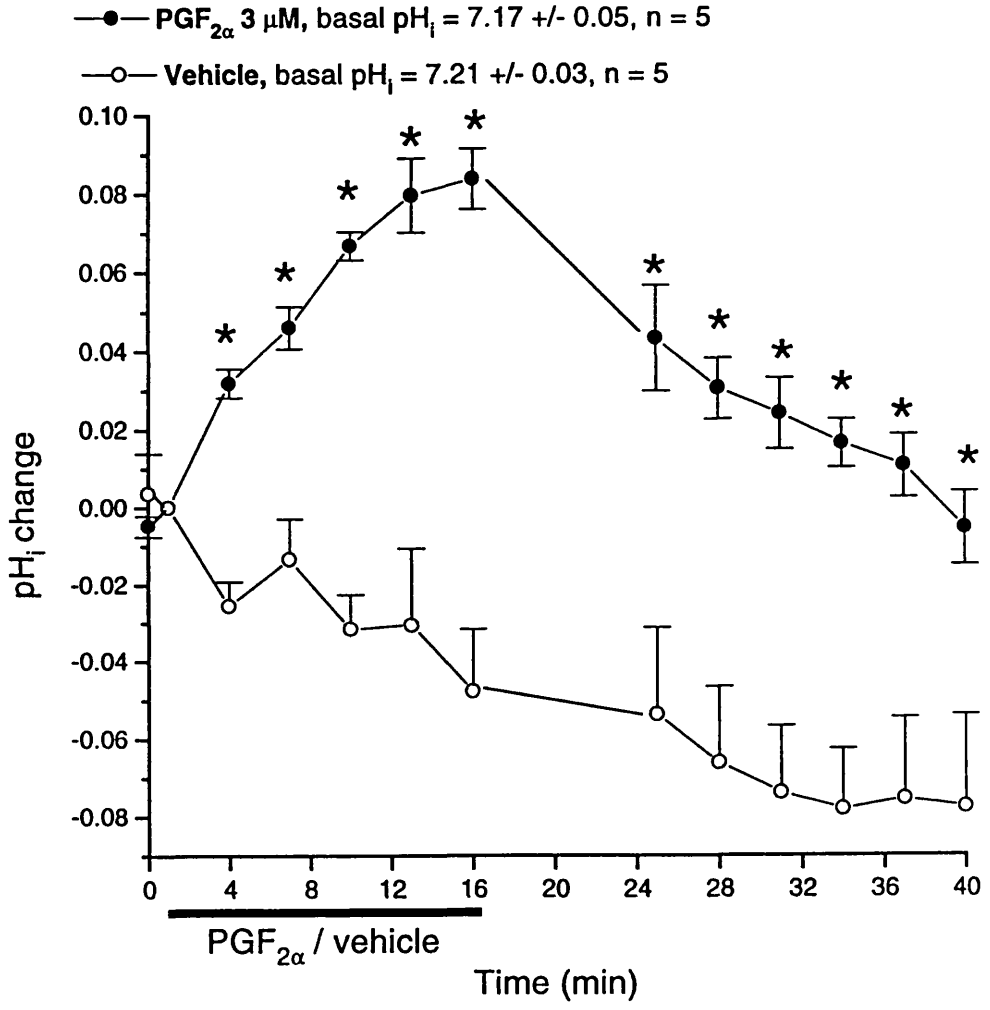
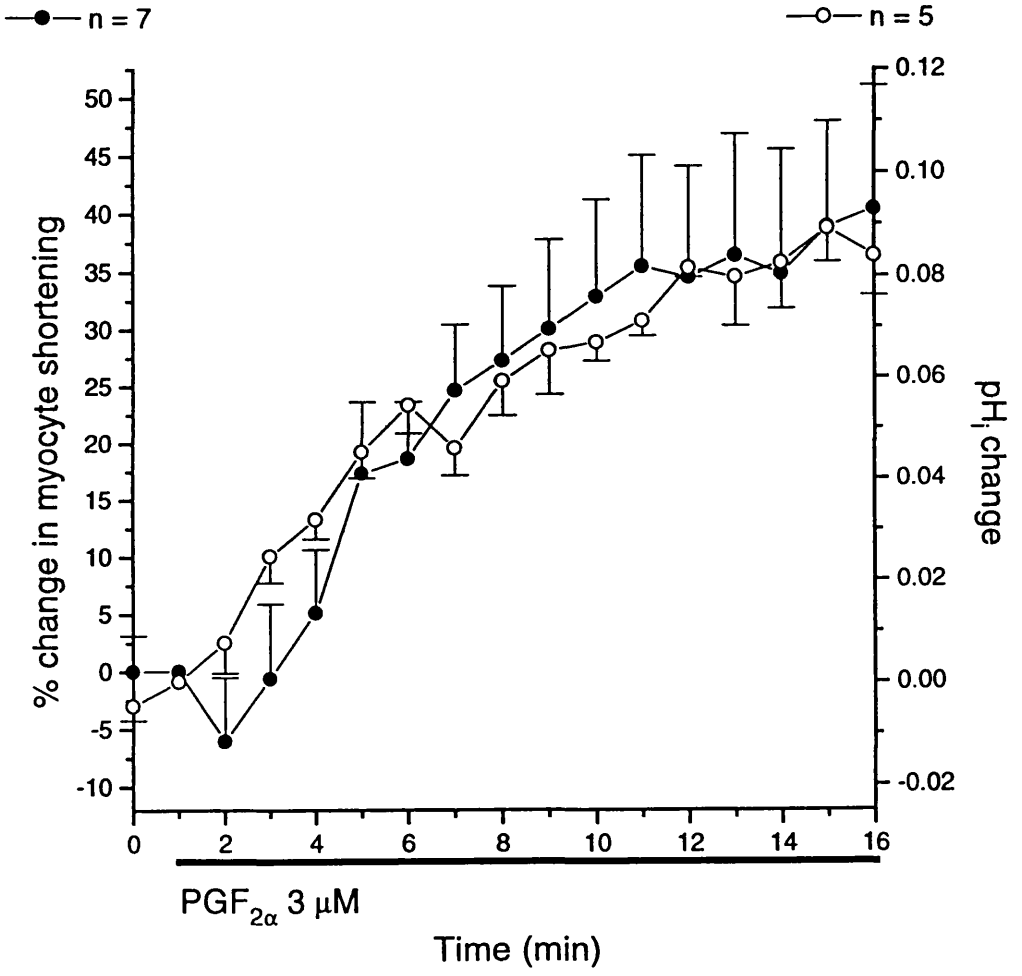


Figure 27. Time course effects of $PGF_{2\alpha}$ (3 μM) on pH_i of BCECF loaded rat ventricular myocytes and shortening of fura-2 loaded rat ventricular myocytes. Myocyte shortening and pH_i was standardised at the start of $PGF_{2\alpha}$ addition.



3.3.5 Effect of inhibitors on the $PGF_{2\alpha}$-induced increase in myocyte shortening and pH_i

The previous section showed that $PGF_{2\alpha}$ increased myocyte pH_i. Therefore, the next sets of experiments were done to find out if this increase in pH_i stemmed from activation of the Na^+/H^+ exchanger and if PKC was involved. HOE 694 was used to inhibit the Na^+/H^+ exchanger (Scholz *et al.*, 1993; Loh *et al.*, 1996), while chelerythrine was used to inhibit PKC (Herbert *et al.*, 1990). Most importantly, whether the $PGF_{2\alpha}$-induced alkalosis could cause the positive inotropy was investigated, since alkalosis is known to sensitise the myofilaments to Ca^{2+} (Fabiato & Fabiato, 1978). In separate sets of experiments, the effects of HOE 694 and chelerythrine on $PGF_{2\alpha}$-induced myocyte shortening were also studied.

a) Effects of HOE 694 on myocyte shortening

The Na^+/H^+ exchanger inhibitor, HOE 694, inhibited the increase in myocyte shortening induced by $PGF_{2\alpha}$ in a concentration dependent manner (figures 28 & 29). HOE 694 at 0.1 μM (a concentration close to the IC_{50} value, Scholz *et al.*, 1993) did not have any effect on basal myocyte shortening, but it reduced the $PGF_{2\alpha}$-induced increase in shortening. At a higher concentration of 10 μM, HOE 694 inconsistently increased myocyte shortening, but abolished that induced by $PGF_{2\alpha}$.

Additionally, washout of 10 μM HOE 694 produced a transient potentiation of myocyte shortening. This effect parallelled a transient rise in pH_i (see 3.3.5b below and figure 30).

The addition of noradrenaline (300 nM) at the end of the experimental protocol increased shortening in all groups, showing that these myocytes had a similar ceiling for shortening (figure 29).

b) Effects of HOE 694 on myocyte pH_i

Figure 30 shows that HOE 694 (10 μM) did not affect pH_i of BCECF-loaded myocytes but it did abolish the alkalosis induced by 3 μM $PGF_{2\alpha}$. Wash out of HOE 694 however, transiently raised pH_i by 0.05 ± 0.02 units (n = 4) after nine minutes,

and a larger increase of up to 0.10 ± 0.01 units (n = 5) was found when $PGF_{2\alpha}$ was also initially present with HOE 694.

c) Effect of chelerythrine on myocyte shortening

Preliminary experiments showed that when chelerythrine was given alone, it augmented myocyte shortening in a concentration dependent manner (2 to 10 μM) while its vehicle, 1 part DMSO to 4000 parts water, did not (figures 31 & 32). Chelerythrine has an IC_{50} value of 0.66 μM for PKC inhibition (Herbert *et al.*, 1990). Therefore, a chelerythrine concentration of 2 μM was chosen for the $PGF_{2\alpha}$ experiments as this was about three times the IC_{50} value but which did not affect basal myocyte shortening.

Myocytes were preselected so that the increase in shortening with rapid pacing (see 2.6.2.4f and 3.3.1c) was similar in both the $PGF_{2\alpha}$ time matched control group and in the group where the PKC antagonist, chelerythrine was added with $PGF_{2\alpha}$ (31.8 ± 3.7% vs. 32.8 ± 5.7% increase, n = 6/group). The shortening response induced by 3 μM $PGF_{2\alpha}$ was significantly less in the presence of 2 μM chelerythrine compared to $PGF_{2\alpha}$ time matched controls (figures 33 & 34). Noradrenaline (300 nM) added at the end of the experimental protocol increased myocyte shortening similarly in all groups (figure 34).

d) Effects of chelerythrine on myocyte pH_i

The PKC inhibitor, chelerythrine, did not modify myocyte pH_i at 2 μM, but the alkalosis induced by 3 μM $PGF_{2\alpha}$ was significantly less in the presence of this concentration of chelerythrine (figure 35). Higher concentrations of chelerythrine were not used as they increased basal myocyte shortening (see 3.3.5c above).

Figure 28. Typical traces of the effects of HOE 694 on the increase in myocyte shortening induced by $PGF_{2\alpha}$ in rat ventricular myocytes. Trace (B) shows that HOE 694 at 0.1 µM reduced the $PGF_{2\alpha}$-induced shortening increase as seen in trace (A), while HOE 694 at 10 µM abolished this effect in trace (C). One of five or six similar experiments per group.

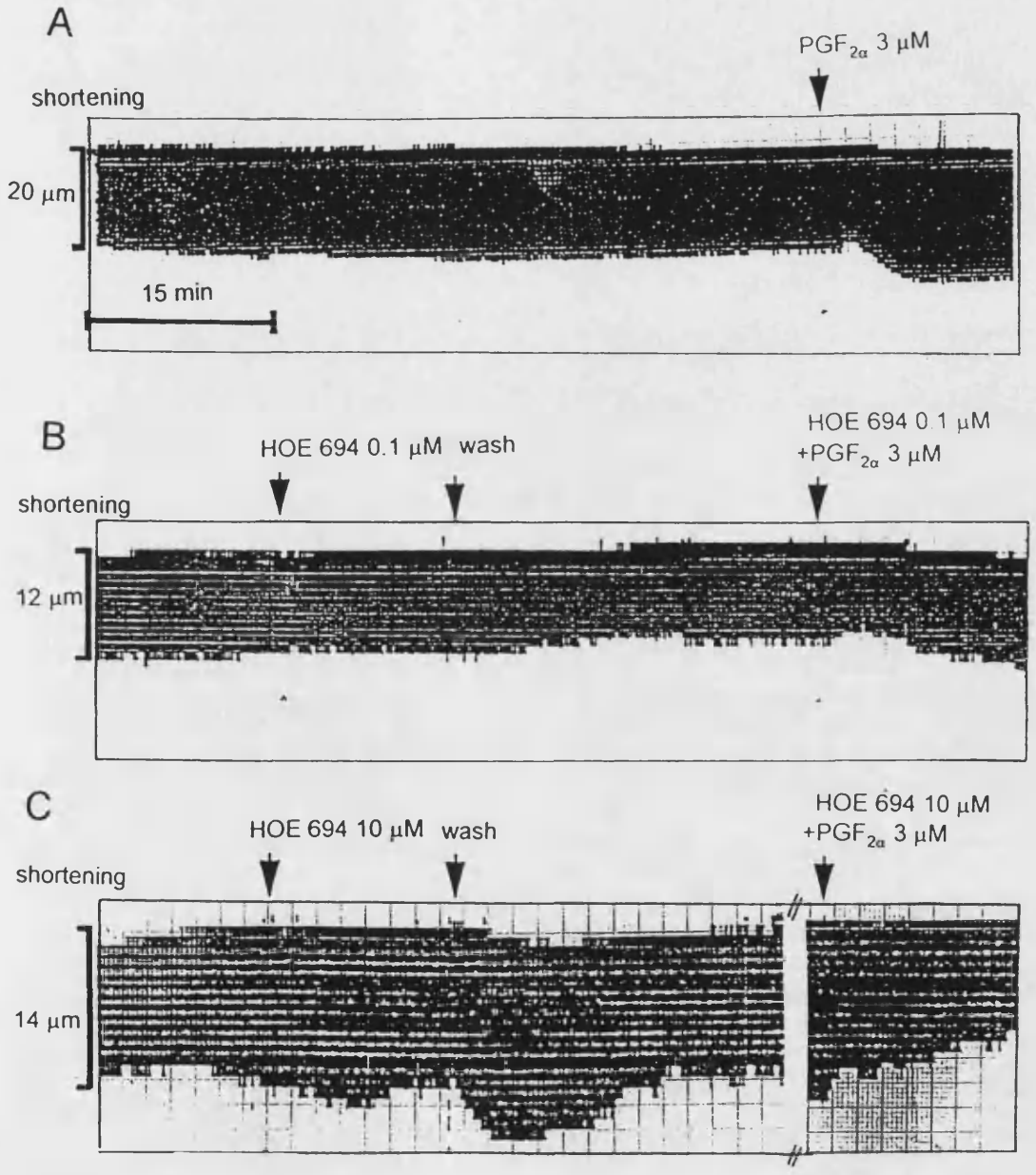


Figure 29. Histogram showing the effects of HOE 694 on the increase in myocyte shortening induced by $PGF_{2\alpha}$ in rat ventricular myocytes. * $P < 0.05$ Wilcoxon signed rank test, # $P < 0.05$ Mann-Whitney U test.

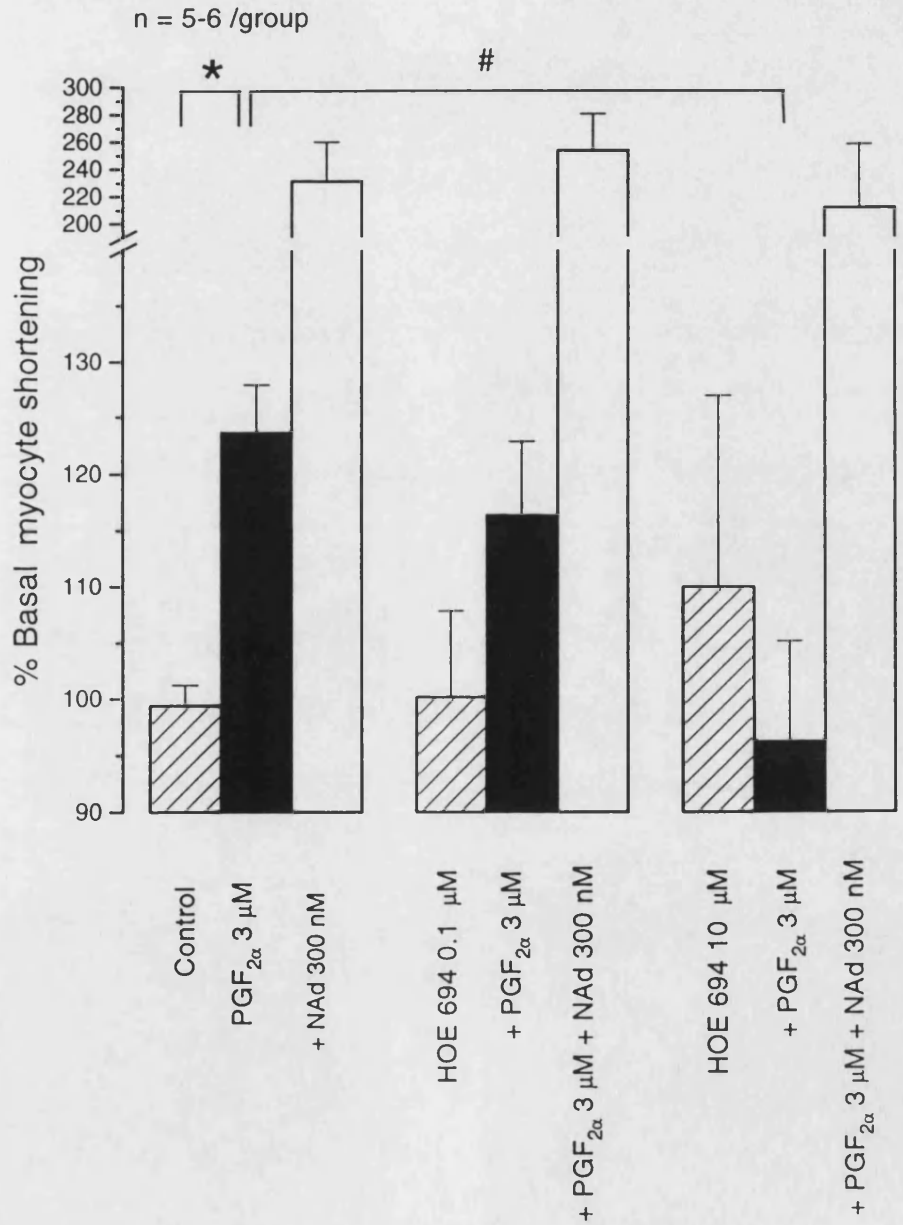


Figure 30. The effect of HOE 694 on the intracellular alkalosis induced by $PGF_{2\alpha}$ in BCECF loaded rat ventricular myocytes. Wash out of HOE 694 was associated with a transient increase in pH_i. pH_i was standardised at the start of drug perfusion. * $P<$ 0.05 (•) vs. (o) , # $P<$ 0.05 (▲) vs. (o), one-way ANOVA.

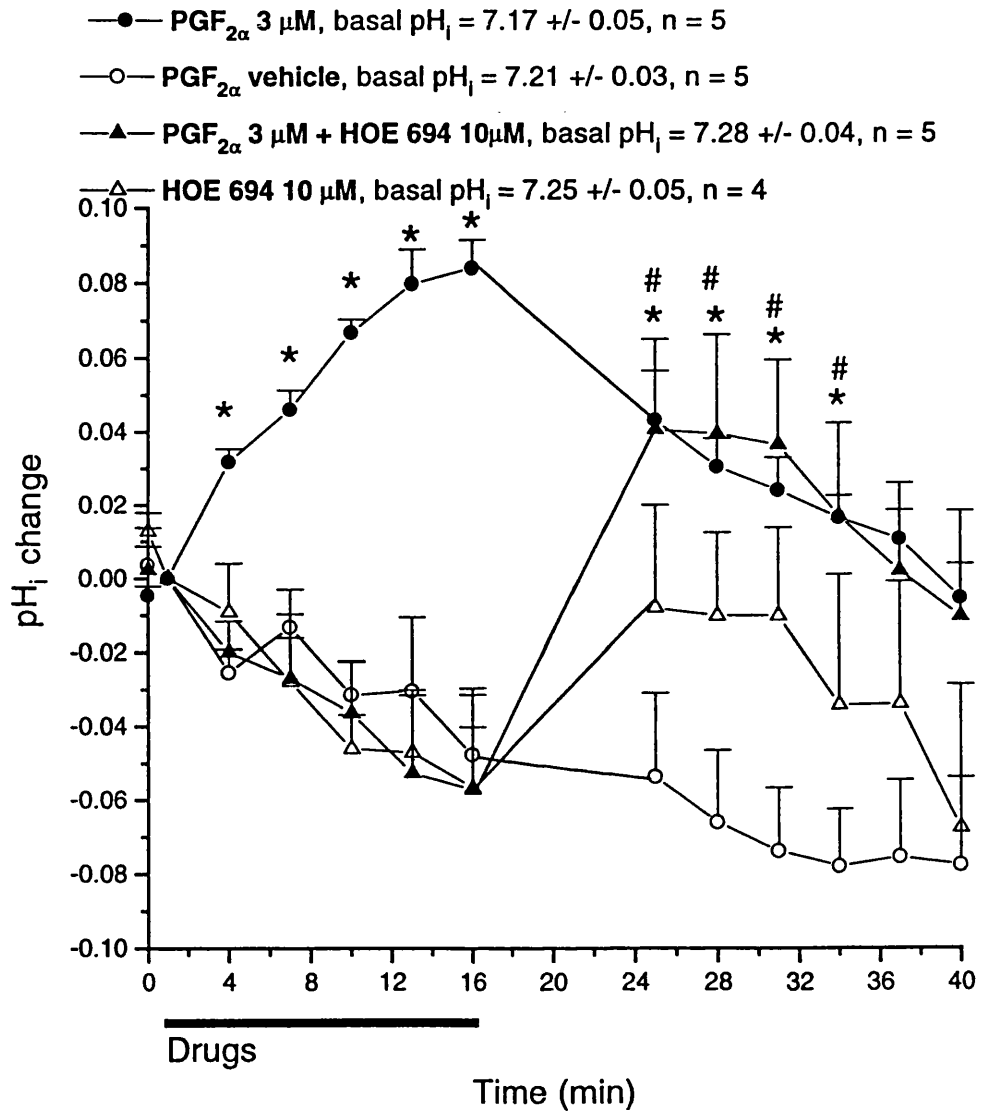


Figure 31. Typical traces showing the effects of chelerythrine and its vehicle, DMSO, on rat ventricular myocyte shortening. Data were obtained after 5 min perfusion of chelerythrine in a non-cumulative manner. One each of 4-14 similar experiments per group.

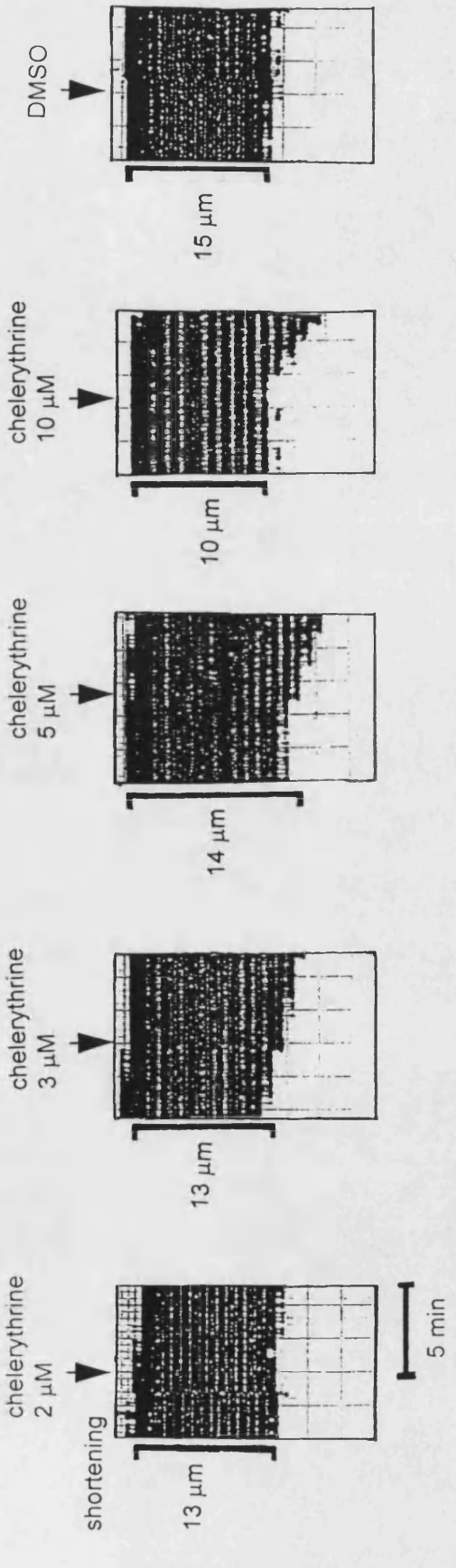


Figure 32. Histogram showing the effects of chelerythrine on rat ventricular myocyte shortening. Data were obtained after 5 min perfusion of chelerythrine in a non-cumulative manner.

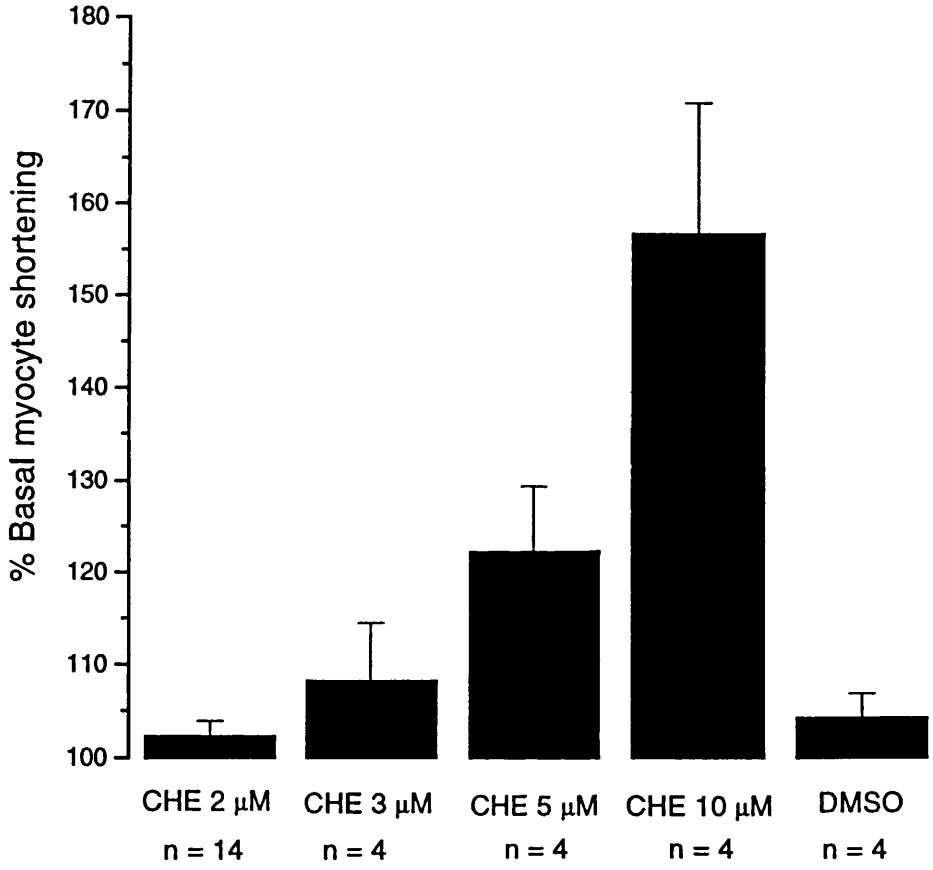


Figure 33. Typical traces showing the effect of chelerythrine on the increase in myocyte shortening induced by $PGF_{2\alpha}$ in rat ventricular myocytes. Trace (A) shows that the vehicle for chelerythrine (DMSO solution) did not affect the $PGF_{2\alpha}$-induced increase in myocyte shortening, while chelerythrine reduced it in trace (B). Chelerythrine alone did not affect basal shortening at 2 μM (C). One of six similar experiments per group.

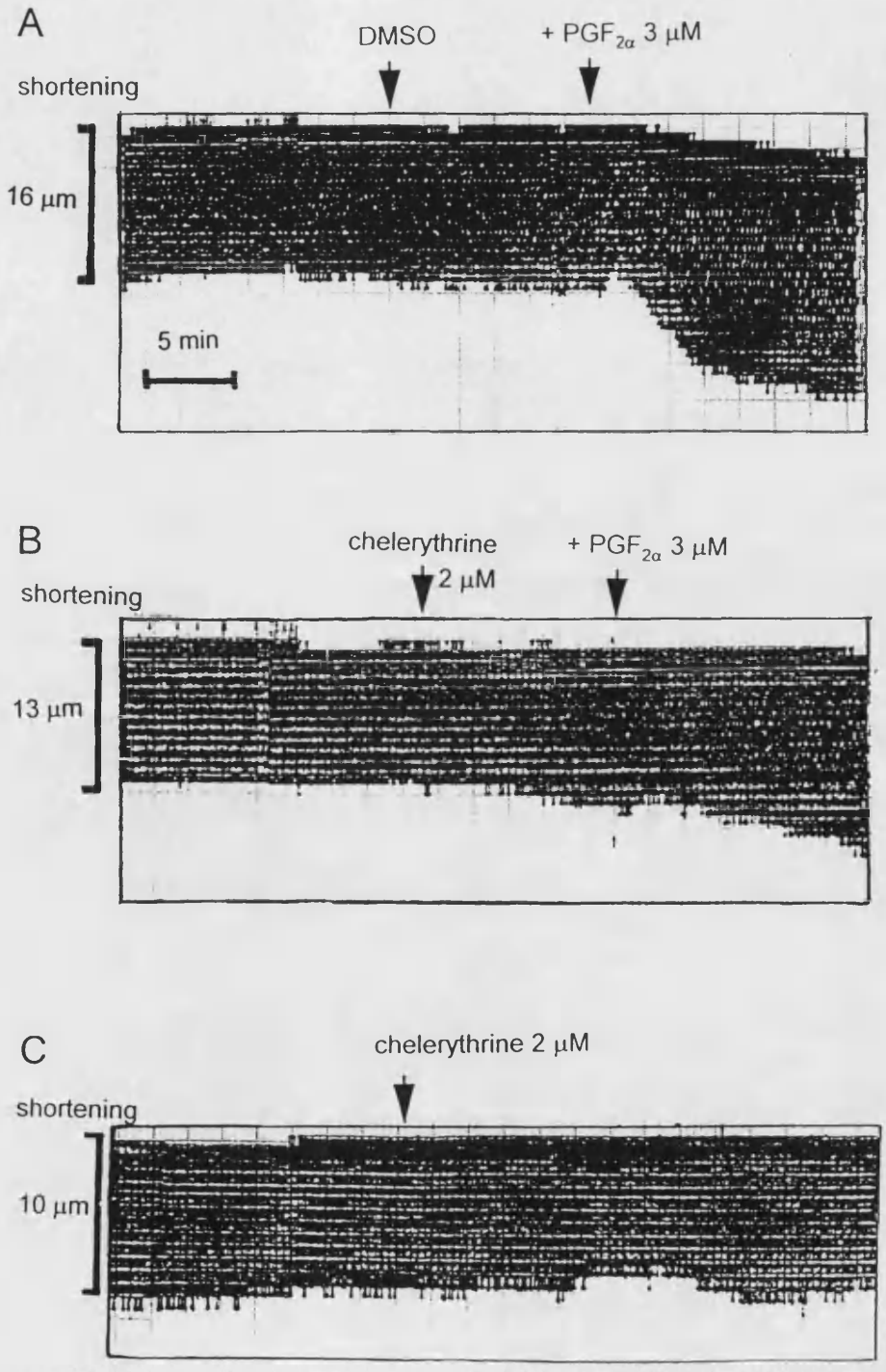


Figure 34. Effects of chelerythrine on the increase in myocyte shortening induced by $PGF_{2\alpha}$ in rat ventricular myocytes. *$P < 0.05$ Wilcoxon signed rank test, # < 0.05 Mann-Whitney U test.

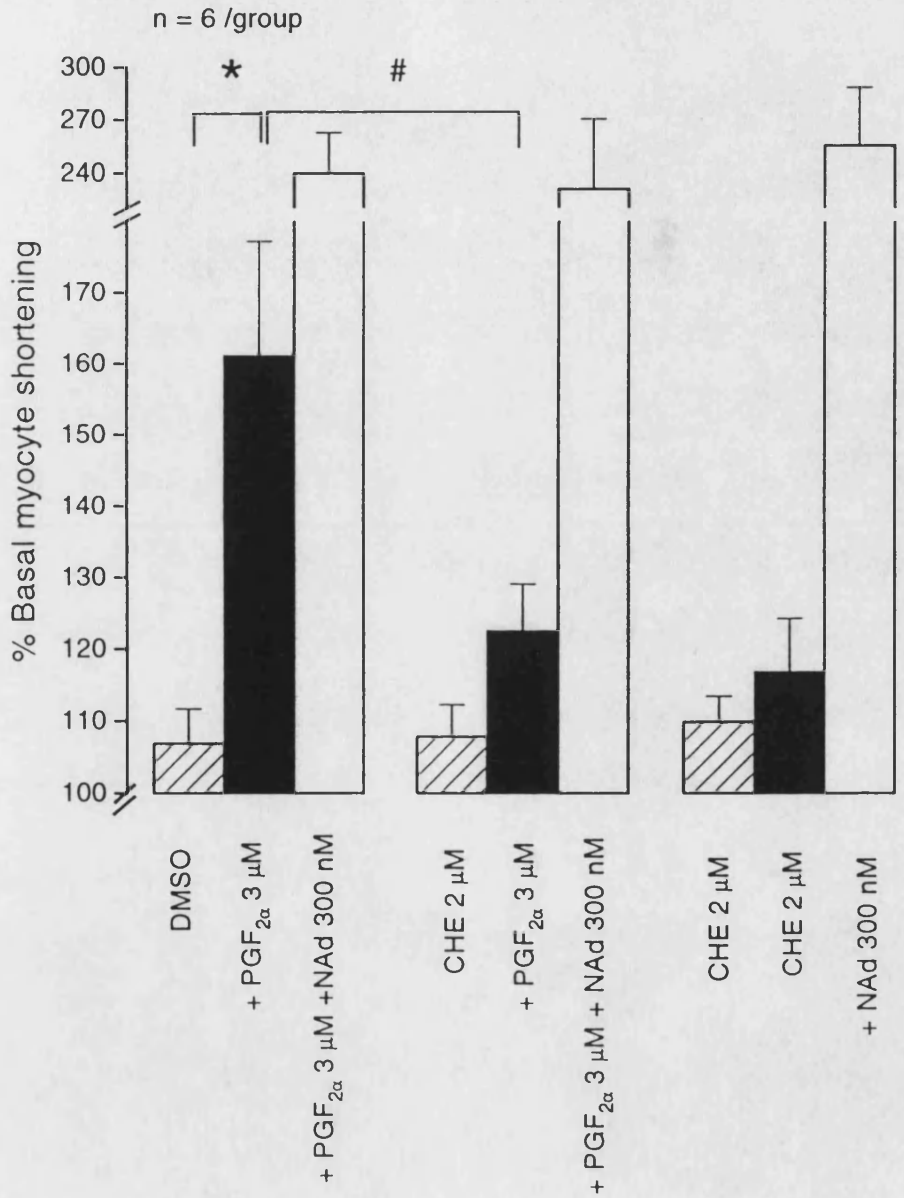
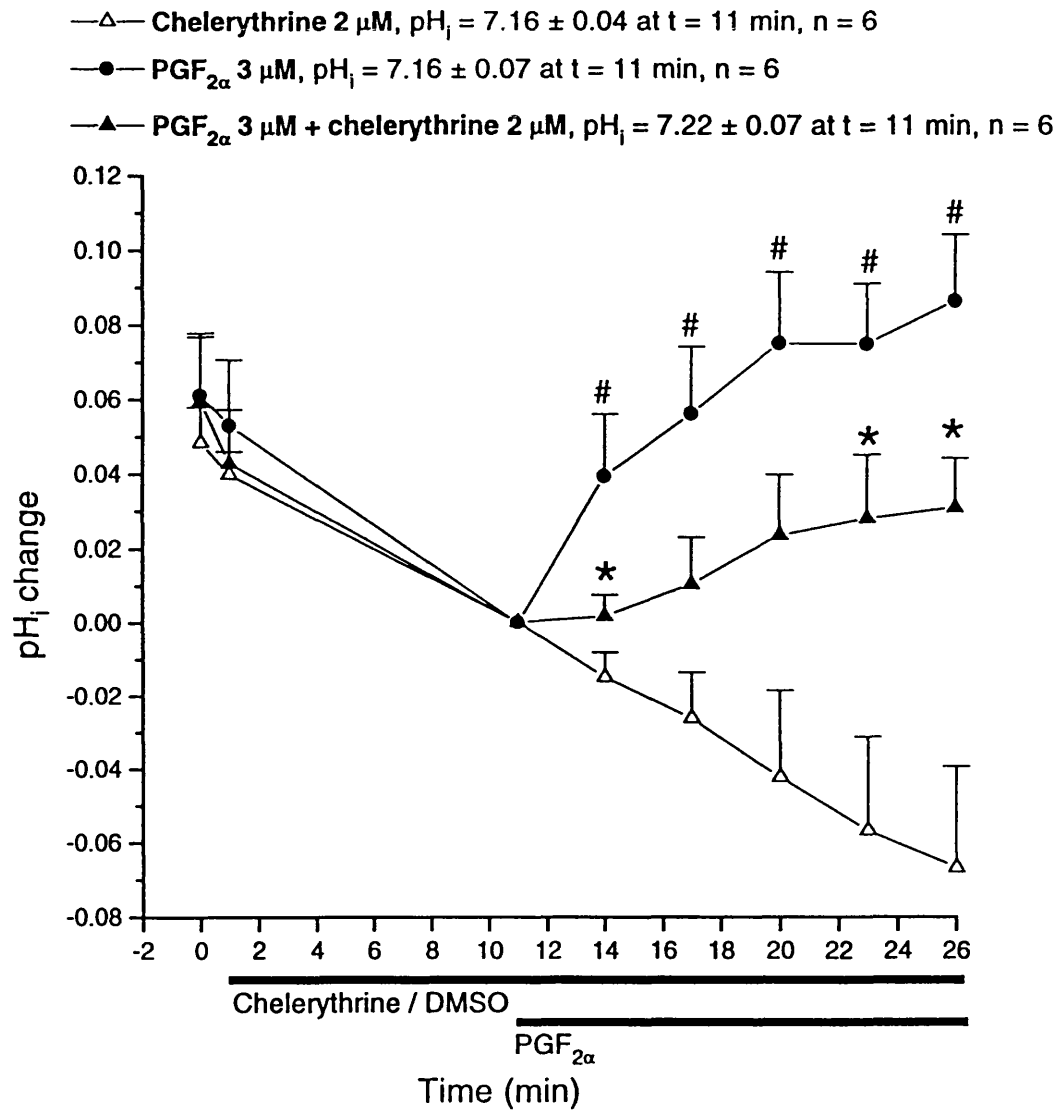


Figure 35. Effect of chelerythrine on the intracellular alkalosis induced by $PGF_{2\alpha}$ in BCECF loaded rat ventricular myocytes. pH_i was standardised at the start of $PGF_{2\alpha}$ addition. # $P < 0.05$ (•) vs. (Δ), * $P < 0.05$ (▲) vs. (•), Kruskal-Wallis test, then Mann-Whitney U test.



SECTION 4

Discussion

4.1 Summary of primary results

The $PGF_{2\alpha}$-induced increase in cardiac contractility could have occurred either by an increase in intracellular Ca^{2+} or by an increase in the sensitivity of the contractile filaments to Ca^{2+}. This study showed that $PGF_{2\alpha}$ increased myocyte shortening without increasing intracellular Ca^{2+} in experiments where Ca^{2+} transients were monitored in fura-2 loaded rat ventricular myocytes. Instead, it appeared to activate the Na^+/H^+ exchanger, as the resulting intracellular alkalosis shown in BCECF loaded myocytes was abolished by the exchanger inhibitor, HOE 694. Since HOE 694 also inhibited the $PGF_{2\alpha}$-induced increase in myocyte shortening, this suggests that the alkalosis sensitised the contractile filaments to Ca^{2+}, leading to the positive inotropic effect.

4.2 Comparison of the effects of cloprostenol and $PGF_{2\alpha}$

$PGF_{2\alpha}$ and cloprostenol increased the contractility of isolated perfused rat hearts, with cloprostenol being more potent than $PGF_{2\alpha}$ (figure 3). Since both these drugs are known agonists of the FP prostaglandin receptor (Coleman *et al.*, 1994), this suggests that the increase in contractility produced by $PGF_{2\alpha}$ may be FP receptor mediated. Furthermore, concentrations as low as 3 nM $PGF_{2\alpha}$ could elicit a positive inotropic effect in atrial and single myocyte preparations (figures 5 & 11). As there are no specific FP receptor antagonists, structure-activity relationships using prostaglandins need to be compared for further evidence that the FP receptor is involved (Kennedy *et al.*, 1982).

The higher potency of cloprostenol compared to $PGF_{2\alpha}$ in perfused rat hearts is consonant to previous results obtained in bovine corpus luteum, where it is attributed to the higher binding affinity of cloprostenol for the FP receptor (Griffin *et al.*, 1997). The higher receptor affinity, in addition to cloprostenol's stability, may also explain why the response to cloprostenol lasted longer than that of $PGF_{2\alpha}$ (figures 1 & 2).

FP receptor activation is associated with an increase in phosphoinositide metabolism. Otani *et al.* (1988) showed that the positive inotropic action of $PGF_{2\alpha}$ correlated with accumulation of inositol phosphates in rat papillary muscles. If this correlation also occurs with cloprostenol in cardiac tissue, then the greater inotropic potency of cloprostenol suggests that even if the Ca^{2+} level is increased, this is not the sole mediator for the positive inotropy. This is deduced from the fact that while cloprostenol is more potent in accumulating inositol phosphates than $PGF_{2\alpha}$ in Swiss 3T3 fibroblasts, both show a similar ability for intracellular Ca^{2+} mobilisation in these cells (Griffin *et al.*, 1997).

4.3 Effects of $PGF_{2\alpha}$ on myocyte shortening and Ca^{2+} transients

Results from experiments with single myocytes in this thesis indicate that the role of Ca^{2+} is not important in mediating the contractile effects of $PGF_{2\alpha}$. $PGF_{2\alpha}$ increased myocyte shortening and decreased diastolic length in a concentration dependent manner. The 40% increase in myocyte shortening and 6% reduction in diastolic cell length in the presence of 3 μM $PGF_{2\alpha}$ (figure 11) was not accompanied by significant changes in the Ca^{2+} transient amplitude nor in the diastolic Ca^{2+} level, as shown in the fura-2 experiments (figure 19).

a) Possible experimental limitations

The lack of effect on the Ca^{2+} transients by $PGF_{2\alpha}$ was not due to compromised viability of the myocytes, as addition of noradrenaline to the myocytes at the end of the protocol could potentiate the amplitude of the Ca^{2+} transients (figure 20). In addition, noradrenaline shortened the duration of the Ca^{2+} transients, but $PGF_{2\alpha}$ did not (figure 21). These effects of noradrenaline are consistent with its ability to activate the β_1-adrenergic receptor, which is coupled to cAMP accumulation and protein kinase A stimulation.

Since fura-2 does buffer Ca^{2+} (Noble & Powell, 1990), there was a possibility that excessive buffering might have affected the outcome of the experiments. Therefore, myocyte shortening was monitored in parallel studies with myocytes loaded with the same concentration of fura-2 (5 μM). These myocytes showed a similar extent of increased shortening to $PGF_{2\alpha}$ as in myocytes not loaded with fura-2 (figures 11 & 14), implying that fura-2 buffering was not important.

Additionally, since $PGF_{2\alpha}$ caused intracellular alkalosis, changes in pH_i also might have affected the binding of Ca^{2+} to fura-2, or affected the excitation spectra of fura-2, thus giving erroneous results when measuring Ca^{2+} transients. When they introduced fura-2 for Ca^{2+} measurement, Grynkiewicz and colleagues (1985) showed that changes in pH from 6.75 to 7.05, at a physiological Ca^{2+} concentration of 200 nM, had little effect on fura-2 signals. In fact, there was a slight inhibition of Ca^{2+} binding to fura-2 by H^+, indicating that any large rise in pH_i would tend to increase

the fura-2 fluorescence intensity. In contrast, Martinez-Zaguilan *et al.* (1991) reported that an increase in pH reduced the 340/380 fluorescence ratio of fura-2 for Ca^{2+}, but they did their in vitro experiments without free Ca^{2+}. Grynkiewicz also obtained similar results without free Ca^{2+}. Regardless, Martinez-Zaguilan also concluded that the pH_i effect on fura-2 is minor when pH_i is more than 7.0. Therefore, it is unlikely that the change in pH of 0.08 units induced by $PGF_{2\alpha}$ would have affected the fura-2 signals to a significant extent.

Lastly, the lack of effect of $PGF_{2\alpha}$ on Ca^{2+} transients was not due to insufficient contact time of the drug with the myocytes. Fura-2 measurements were recorded only up to seven minutes, while the $PGF_{2\alpha}$-induced increase in myocyte shortening and alkalosis stabilised around ten minutes. However, this increase in shortening and alkalosis were statistically significant even at seven minutes, in contrast to the Ca^{2+} measurements. It would have been ideal to have measured Ca^{2+} transients for a longer period, but these experiments preceded that of the myocyte shortening experiments due to unavailability of equipment then. The time chosen for Ca^{2+} transient measurements was based on results from the whole hearts, where the $PGF_{2\alpha}$-induced increase in contractility was maximum within five to seven minutes at the highest dose (figure 1).

b) Effects of pH_i on Ca^{2+} transients

The results from the fura-2 studies showing $PGF_{2\alpha}$'s lack of effect on Ca^{2+} transients are not entirely unexpected. Other agonists that increase myocyte contractility such as phenylephrine, angiotensin II and endothelin-1 also show similar effects in some experiments (Gambassi *et al.*, 1992; Terzic *et al.*, 1992; Kohmoto *et al.*, 1993; Ikenouchi *et al.*, 1994), but show a slight increase in Ca^{2+} levels in other experiments (Fedida & Bouchard, 1992). These agonists and $PGF_{2\alpha}$ have in common the fact that they activate the G_q-phospholipase C signalling pathway. In addition, phenylephrine, angiotensin II and endothelin-1 have also been shown to increase intracellular pH as part of their mechanisms of action (Kramer *et al.*, 1991; Gambassi *et al.*, 1992; Kohmoto *et al.*, 1993).

However, when the effects of pH changes alone are taken into account, the $PGF_{2\alpha}$-induced alkalosis would be expected to *decrease* Ca^{2+} transient amplitude. Allen and Orchard (1983) have shown that alkalosis induced by decreasing CO_2 in the buffer decreases systolic Ca^{2+}, as measured by aequorin light emission in rat papillary muscles. Consistent with this result in the reverse situation, acidosis increases Ca^{2+} transients (Allen & Orchard, 1983; Ward & Moffat, 1995). The fura-2 data presented in this thesis showed no significant change in Ca^{2+} transient amplitude with $PGF_{2\alpha}$, even though pH_i was clearly increased in the BCECF experiments. This suggests that $PGF_{2\alpha}$ or its second messengers may have direct actions that increase free intracellular Ca^{2+}, and this would oppose the direct effect of the alkalosis. The apparent lack of effect of $PGF_{2\alpha}$ on the Ca^{2+} transients may also be explained by its modulation of the balance between Ca^{2+} influx and Ca^{2+} extrusion.

c) Sarcoplasmic reticulum Ca^{2+} stores

Unlike some of the other prostaglandins, $PGF_{2\alpha}$ does not activate cAMP in cardiac muscle (Metsa-Ketela, 1981; Otani *et al.*, 1988), so this would rule out protein kinase A-induced Ca^{2+} fluxes. Instead, $PGF_{2\alpha}$ activates the phosphoinositide cascade in rat hearts (Otani *et al.*, 1988). This results in accumulation of IP_3, which could potentially increase Ca^{2+} release from the sarcoplasmic reticulum, as it does in smooth muscle cells. However, Otani showed that this $PGF_{2\alpha}$-induced IP_3 increase was transient (maximum at 30 s) and it did not correspond to the time course of the increase in contractility (up to 7 min). Additionally, localisation of the IP_3 receptors in cardiac myocytes does not favour sarcoplasmic Ca^{2+} release. Kijima *et al.* (1993) showed that IP_3 receptors were localised in the intercalated discs in rat atrial and ventricular myocytes, and there was little or no IP_3 binding in the sarcoplasmic reticulum.

However, Otani *et al.* (1986) and Sabatini-Smith (1970) suggested that in guinea-pig hearts, the $PGF_{2\alpha}$-induced increase in contractility was dependent on increased Ca^{2+} release from the sarcoplasmic reticulum. Otani used procaine and TMB-8 (10 μM) as inhibitors of sarcoplasmic reticulum Ca^{2+} release, but these inhibitors do have additional effects. Procaine blocks sarcolemmal Na^+ channels, while TMB-8 inhibits

Na^+/Ca^+ and Na^+/H^+ exchange at concentrations above 2 μM in rat atria (Northover, 1992), and inhibits sarcolemmal Ca^{2+}, Na^+ and K^+ channels in guinea-pig ventricular myocytes at 10 μM (Himmel & Ravens, 1996a,b). Therefore, these non-specific effects of TMB-8 render the results from Otani's study inconclusive. Sabatini-Smith (1970) did, however, find that $PGF_{2\alpha}$ increased Ca^{2+} accumulation in the guinea-pig fragmented sarcoplasmic reticulum by measurements of $^{45}Ca^{2+}$. This may be a direct stimulatory effect of $PGF_{2\alpha}$, in addition to the possible stimulation of sarcoplasmic reticulum Ca^{2+} loading by alkalosis in cardiac tissue (Fabiato & Fabiato, 1978; Orchard, 1987).

d) L-type Ca^{2+} current

A potential source of Ca^{2+} availability for the myofilaments is Ca^{2+} influx through L-type Ca^{2+} channels. Otani *et al.* (1988) reported that $PGF_{2\alpha}$ initiated contractions of 25 mM K^+ depolarised, electrically stimulated rat papillary muscles, and that this effect was blocked by the L-type Ca^{2+} channel inhibitor, nifedipine. The concentration of nifedipine used was 0.1 μM, a concentration that should not have unspecific effects. However, this experiment may not reflect the effect of $PGF_{2\alpha}$ at normal physiological K^+ concentrations. This is because the initiation of muscle contractions, believed to be due to L-type Ca^{2+} channel activation, occurred over a time course clearly different to that of the positive inotropic action observed with normal K^+ concentrations in the buffer. In addition, Otani *et al.* (1986) reported that the $PGF_{2\alpha}$-induced positive inotropy in guinea-pig atria was not inhibited by the Ca^{2+} channel antagonist, verapamil.

In rabbit ventricular myocytes, stimulation of the L-type Ca^{2+} current by angiotensin II is dependent on Na^+-H^+ exchanger activation (Kaibara *et al.*, 1994). Since $PGF_{2\alpha}$ stimulated the Na^+-H^+ exchanger in my study, $PGF_{2\alpha}$ might be expected to increase intracellular Ca^{2+} by L-type Ca^{2+} channel activation. However, in our laboratory, Reeves showed that $PGF_{2\alpha}$ did not affect the activity of L-type Ca^{2+} channels in patch clamp experiments in rat ventricular myocytes (figure 22). She used 100 nM $PGF_{2\alpha}$, a concentration that showed marked enhancement of myocyte shortening in this thesis.

Metsa-Ketela (1981) has measured prostaglandin-induced $^{45}Ca^{2+}$ accumulation in rat atrial preparations. In that study, prostaglandins $F_{2\alpha}$, E_2, E_1, and I_2 caused atria to accumulate $^{45}Ca^{2+}$ similarly, but the increase in atrial contractility was highest with $PGF_{2\alpha}$. The lack of correlation between $^{45}Ca^{2+}$ accumulation and contractility suggests that Ca^{2+} may not be important in mediating the positive inotropy of $PGF_{2\alpha}$. The concentration of $PGF_{2\alpha}$ used in the $^{45}Ca^{2+}$ experiment described was 50 μM, fifty times higher than the concentration required for maximal response in the same tissue preparation in my study. As $PGF_{2\alpha}$ is a lipid, this high concentration may have non-specific effects on the cell membrane such as to increase its permeability and allow $^{45}Ca^{2+}$ entry into the cell.

e) Na^+/Ca^{2+} exchange

Karmazyn *et al.* (1981) reported that $PGF_{2\alpha}$ inhibited the Na^+/K^+ ATPase activity of rat sarcolemmal vesicles. This would result in increased intracellular Na^+ and decreased gradient for Ca^{2+} expulsion through the Na^+/Ca^{2+} exchanger. Therefore, Ca^{2+} would accumulate in the cell. It would seem likely that stimulation of the Na^+/H^+ exchanger by $PGF_{2\alpha}$ seen in this thesis would also cause intracellular Na^+ and Ca^{2+} to accumulate. However, Na^+ loading (Rasmussen *et al.*, 1989) and alkalosis (reviewed by Orchard & Kentish, 1990) also activate Na^+ pumping of the Na^+/K^+ ATPase, as opposed to the direct inhibition of the ATPase by $PGF_{2\alpha}$ as observed by Karmazyn. Any potential changes in the Na^+ gradient may therefore be insufficient to activate Ca^{2+} influx through the Na^+/Ca^{2+} exchanger. Additionally, the alkalosis induced by $PGF_{2\alpha}$ may also stimulate Ca^{2+} efflux through this exchanger (Philipson *et al.*, 1982; Dipolo & Beauge, 1982).

f) Ca^{2+} transient decay

In my study, the rate of decay of the Ca^{2+} transient was unchanged in the presence of $PGF_{2\alpha}$ (figure 21). Intracellular alkalosis should *increase* the rate of Ca^{2+} transient decay (Allen & Orchard, 1983) as an increase in the Ca^{2+} affinity of troponin C, coupled with sarcoplasmic reticulum Ca^{2+} pumping, should reduce free Ca^{2+} more quickly (Lee & Allen, 1996). If $PGF_{2\alpha}$ did have other effects that increase free Ca^{2+} as discussed above, then this would explain why no change in the rate of decline was

detected. Another hypothesis would be that the $PGF_{2\alpha}$-induced alkalosis did not increase the affinity of troponin C for Ca^{2+}, instead it increased maximum Ca^{2+} activated force (discussed later in 4.8).

To summarise section 4.3, there is a possibility that $PGF_{2\alpha}$ stimulates the sarcoplasmic reticulum Ca^{2+} loading, and inhibits the Na^{+}/K^{+} ATPase, but the alkalosis *per se* induced by $PGF_{2\alpha}$ offsets any potential Ca^{2+} increase, so that the Ca^{2+} transient amplitude appears unchanged.

4.4 Effect of $PGF_{2\alpha}$ on pH_i

While the fura-2 experiments showed that $PGF_{2\alpha}$ did not change the Ca^{2+} transients, experiments using myocytes loaded with the pH indicator, BCECF, indicated that $PGF_{2\alpha}$ clearly increased myocyte intracellular pH (figure 26). This alkalosis occurred over a time course similar to that of the increase in myocyte shortening and was reversible upon washout of the drug. This increase in pH of up to 0.08 units is comparable to that induced by other positive inotropic agents. Gambassi *et al.* (1992) recorded an increase of 0.06 pH units with phenylephrine in rat ventricular myocytes; Kramer *et al.* (1991) and Kohmoto *et al.* (1993) measured an increase of 0.13 and 0.08 pH units with endothelin-1 in rat and rabbit ventricular myocytes respectively; while Ikenouchi *et al.* (1994) observed an increase of 0.2 pH units with angiotensin II in rabbit ventricular myocytes.

The $PGF_{2\alpha}$-induced intracellular alkalosis is likely to be due to activation of the Na^+/H^+ exchanger because the Na^+/H^+ exchanger inhibitor, HOE 694, abolished this alkalosis (figure 30). Loh *et al.* (1996) showed that HOE 694 was selective for NHE-1, the ubiquitous Na^+/H^+ exchanger isoform, over other sarcolemmal pH regulating mechanisms in guinea-pig myocytes. The concentration of HOE 694 used in my study was 10 μM, close to the concentration of 30 μM for maximal inhibition of the exchanger in guinea-pig myocytes. It did not affect basal intracellular pH with respect to time matched controls, consistent with the fact that the Na^+/H^+ exchanger is quiescent or minimally activated under basal conditions (Wallert & Frohlich, 1989), and therefore ruling out physiological antagonism of the $PGF_{2\alpha}$-induced alkalosis. The control myocyte group did, however, show a gradual decline in pH_i over the time course of the experiment. This effect has also been observed by Sun *et al.* (1996). As the $PGF_{2\alpha}$-induced pH_i change of 0.08 units is the maximum difference compared to pre-drug value, this value is an underestimate when compared to time matched controls. When compared to time matched controls, $PGF_{2\alpha}$ increased pH_i by 0.13 units (figure 30).

4.5 The role of alkalosis in the positive inotropic effect of $PGF_{2\alpha}$

Does the $PGF_{2\alpha}$-induced positive inotropic effect depend on the alkalosis? It appears that this is so, as HOE 694 also eliminated the increase in myocyte shortening at the same concentration that abolished the alkalosis.

HOE 694 itself, at a concentration of 10 μM, sometimes increased basal myocyte shortening (figure 29). Scholz *et al.* (1993) also observed this HOE 694-induced contractile action in ischaemic rat working hearts. Therefore, to avoid complications in interpretation of the data, the effects of HOE 694 alone and in combination with $PGF_{2\alpha}$ were always tested in the same myocyte. This basal effect of HOE 694 on myocyte shortening was not due to changes in pH_i, as checked in pH_i experiments using BCECF loaded myocytes (figure 30). Other non-specific Na^+/H^+ exchanger inhibitors, such as amiloride, also inhibit the Na^+/Ca^{2+} exchanger (Lazdunski *et al.*, 1985). Since HOE 694 has some structural similarity to amiloride, it may also have similar effects. However, results from Hendrikx *et al.* (1994) suggest that it is unlikely that 1 μM HOE 694 inhibits the Na^+/Ca^{2+} exchanger in isolated perfused rat hearts. In contrast, HOE 694 appears to inhibit Na^+ channels (Legrand *et al.*, 1996), but this would reduce basal myocyte shortening, and not increase it.

This increase in myocyte shortening with HOE 694 implies that the slight decline in shortening of 3% occurring in the presence of both $PGF_{2\alpha}$ and HOE 694 is an underestimate of a negative inotropic effect uncovered when the $PGF_{2\alpha}$-induced positive inotropy was removed. The actual figure under those conditions is about 13% (figure 29). This $PGF_{2\alpha}$-induced negative inotropic effect is discussed further in section 4.8.

This thesis showed a correlation between the amplitude of the positive inotropic effect induced by $PGF_{2\alpha}$ and that induced by increasing stimulation frequency from 1 Hz to 2 Hz (figure 16). Such evidence may also support the involvement of the Na^+/H^+ exchanger in the $PGF_{2\alpha}$-induced positive inotropy. The change in contractility with stimulation rate is known as a 'staircase' effect. An increased contractility, or positive staircase, is thought to be due to enhanced Ca^{2+} entry

through L-type Ca^{2+} channels (Borzak *et al.*, 1991) and the Na^+/Ca^{2+} exchanger, and therefore increased sarcoplasmic reticulum Ca^{2+} loading (Frampton *et al.*, 1991). Additionally, Na^+ loading occurs during a positive staircase (Frampton *et al.*, 1991).

More Na^+ goes into the cell through Na^+ channels during faster stimulation rates. Additionally, because of the concentration gradient across the sarcolemma, larger Na^+ influx occurs in myocytes with lower cytosolic Na^+ levels, rather than in those with higher Na^+ levels. The enhanced depolarisation of the membrane then stimulates more Ca^{2+} influx through Ca^{2+} channels and Na^+/Ca^{2+} exchange. This should result in a larger positive staircase in myocytes with lower cytosolic Na^+. Similarly, if the $PGF_{2\alpha}$-induced alkalosis is due to activation of the Na^+/H^+ exchanger, then the extent of alkalosis would also depend on the Na^+ gradient across the sarcolemmal membrane, in the absence of intracellular Ca^{2+} changes. Therefore, the lower the cytosolic Na^+ concentration, the higher the Na^+ influx through the Na^+/H^+ exchanger, and the more protons extruded, the larger the resultant alkalosis and the $PGF_{2\alpha}$-induced positive inotropy.

The explanation for the correlation between the change in contractility induced by $PGF_{2\alpha}$ and rapid pacing being dependent on Na^+ loading is only hypothetical. However, this principle seems to apply to myocytes that show a negative staircase. In these myocytes, Na^+ loading is less, and there is little change in cytosolic Ca^{2+}, compared to myocytes displaying a positive staircase (Frampton *et al.*, 1991). One example where this hypothesis could be extended is to hypertrophied guinea-pig left ventricular myocardium. This preparation does not show a positive staircase effect, and it has a characteristically higher intracellular Na^+ content when compared to non-hypertrophied myocardium (Gray *et al.*, 1997).

With 'normal' cells, the percentage of myocytes that showed a positive staircase in fifty randomly chosen myocytes in my study was 68%, while 8% showed a flat staircase, and 24% showed a negative staircase (decreased shortening). These values are similar to that found by Frampton *et al.* (1991). They reported that under similar

conditions of room temperature and stimulation from 1 Hz, 60% of myocytes showed a positive staircase, while the others showed a flat or negative staircase.

The observation that there was correlation between the positive inotropy induced by $PGF_{2\alpha}$ and that by rapid pacing was used towards the end of the project, to ensure that myocytes of the different protocol groups could potentially respond similarly to $PGF_{2\alpha}$. All myocytes used in the experiment testing the involvement of PKC in the $PGF_{2\alpha}$-induced increase in myocyte shortening were tested for their staircase effects early in the protocol. Myocytes were preselected so that the increase in shortening with rapid pacing was similar in both control myocyte group and in the group where the PKC antagonist, chelerythrine, was used (see 3.3.5c). The higher response to $PGF_{2\alpha}$ in the control group shown in figure 33 when compared to that of figure 11A may be explained by this selection method, which is biased towards myocytes with a positive staircase.

4.6 The role of PKC in the positive inotropic effect of $PGF_{2\alpha}$

Since it appeared that $PGF_{2\alpha}$ stimulated the Na^+/H^+ exchanger and increased myocyte shortening, the role of PKC in these effects was investigated. It is questionable if PKC can phosphorylate the Na^+/H^+ exchanger in all types of myocytes. While rat NHE-1 contains phosphorylation sites for PKC (Kandasamy *et al.*, 1995), rabbit cardiac NHE may not (Fliegel *et al.*, 1992). This effect may be species dependent, as my study supports the idea that PKC does activate the Na^+/H^+ exchanger. The PKC inhibitor, chelerythrine, significantly attenuated both the $PGF_{2\alpha}$-induced alkalosis and positive inotropic effect (figures 34 & 35). The chelerythrine concentration used was 2 μM, above the reported IC_{50} of 0.66 μM for PKC inhibition (Herbert *et al.*, 1990). The specificity of this drug with regards to cardiac cellular mechanisms other than protein kinases is largely unknown, but chelerythrine has been reported to inhibit guinea-pig brain Na^+/K^+ATPase at 100 μM (Cohen *et al.*, 1978). If this also occurs in cardiac myocytes, then high concentrations of chelerythrine would cause Ca^{2+} accumulation and increase myocyte contractility. Indeed, higher concentrations above 2 μM could not be used as chelerythrine was found to consistently increase basal contractility in a concentration dependent manner (figure 32).

In view of the possible lack of specificity of chelerythrine, whether PKC is solely responsible for the $PGF_{2\alpha}$-induced activation of the Na^+/H^+ exchanger cannot be concluded. The Na^+/H^+ exchanger is also regulated by Ca^{2+}-calmodulin dependent protein kinase (Fliegel *et al.*, 1992; Le Prigent *et al.*, 1997), members of the MAP kinase family and those activated by extracellular signal-regulated (ERK) kinases (Takahashi *et al.*, 1997), or by a closely associated regulator protein (Noel & Pouyssegur, 1995). Since $PGF_{2\alpha}$ did not change Ca^{2+} transients in this thesis, it is unlikely that Ca^{2+}-calmodulin dependent protein kinase activated the Na^+/H^+ exchanger. Other than the phospholipase C pathway, $PGF_{2\alpha}$ activates tyrosine kinase and MAPK cascade in NIH-3T3 cells (Watanabe *et al.*, 1994; Watanabe *et al.*, 1995), suggesting that the Na^+/H^+ exchanger may be activated by member(s) of the MAPK cascade. In vascular smooth muscle cells, angiotensin II activates a 90 kDa protein termed $p90^{rsk}$, which phosphorylates NHE-1 and is dependent on ERK1/2 kinase (Takahashi *et al.*, 1997). Also, although activation of the MAPK cascade may

be more important for cell proliferation, there is a possibility that it can regulate contraction. The $PGF_{2\alpha}$-induced rat puerperal uterine contraction depends partly on Ca^{2+} mobilisation and partly on MAPK kinase activity (Ohmichi *et al.*, 1997). Inhibition of MAPK kinase with PD098059 attenuates the $PGF_{2\alpha}$-induced uterine contraction without affecting Ca^{2+} mobilisation. So, perhaps $PGF_{2\alpha}$ also depends on MAPK kinase or some other kinase, in addition to PKC, to activate the Na^{+}/H^{+} exchanger.

4.7 Effects of alkalosis on the contractile proteins

As discussed, the positive inotropic effect induced by $PGF_{2\alpha}$ is probably not due to an increase in intracellular Ca^{2+}, but is associated with an increase in intracellular pH. Fabiato & Fabiato (1978) showed that alkalosis increased the affinity of troponin C for Ca^{2+}, and it also increased the maximum Ca^{2+} activated force of skinned rat papillary muscles. Such effects on myofilament Ca^{2+} sensitivity could account for the positive inotropy of $PGF_{2\alpha}$. It would also explain the $PGF_{2\alpha}$-induced reduction in myocyte diastolic length in the absence of changes in diastolic Ca^{2+} (Spurgeon *et al.*, 1992). One example in which $PGF_{2\alpha}$ has been shown to sensitise the myofilaments to Ca^{2+} is vascular smooth muscle (Suematsu *et al.*, 1991, Hori *et al.*, 1993).

For further evidence that $PGF_{2\alpha}$ sensitises the cardiac myofilaments to Ca^{2+}, I plotted a phase-plane diagram according to Spurgeon *et al.* (1992). $PGF_{2\alpha}$ shifted the late relaxation phase of the fura-2/myocyte length loop upward and leftward of the control loop, indicating an enhanced myofilament sensitivity to Ca^{2+} (figure 23). This method, however, suffers from drawbacks in my study because the average myocyte length and fura-2 fluorescence were obtained from separate experiments. Furthermore, the synchronisation of the twitch and Ca^{2+} could not be accurately defined.

However, abolishing the $PGF_{2\alpha}$-induced alkalosis with HOE 694 did abolish the increase in myocyte shortening. This means that the positive inotropic effect was most probably due to the alkalosis, as the only established means by which this could occur without Ca^{2+} changes was by increasing the affinity of troponin C for Ca^{2+} and by increasing the maximum Ca^{2+} activated force.

4.8 $PGF_{2\alpha}$ -induced negative inotropic action

Brenner *et al.* (1987) reported that concentrations of 1 μM to 100 μM $PGF_{2\alpha}$ reduced the contractility of cultured neonatal rat ventricular myocytes. The possibility that $PGF_{2\alpha}$ may also have a negative inotropic effect in adult ventricular myocytes is deduced from observations during the course of the experiments. Firstly, when the increase in myocyte shortening induced by $PGF_{2\alpha}$ was inhibited by HOE 694, a reduction in myocyte shortening was observed (figures 28C & 29). Secondly, single ventricular myocytes occasionally showed an initial transient reduction in shortening before the positive inotropic effect of $PGF_{2\alpha}$ developed (figures 28A & 33A).

The depressant action of $PGF_{2\alpha}$ may be due to selective activation of PKC isozymes. PKCδ and PKCε phosphorylation of troponin I decreases the activity of actinomyosin MgATPase, and may desensitise the cardiac filaments to Ca^{2+} (Noland & Kuo, 1991; Jideama *et al.*, 1996). On the other hand, PKC may reduce cardiac contractility by reducing Ca^{2+} transients (Capogrossi *et al.*, 1990). Kissling *et al.* (1997) reported an example of receptor mediated negative inotropy where PKC could have been involved. In their study, activation of the α-adrenergic receptor by low doses of adrenaline reduced the contractility of rat papillary muscles; this effect was abolished by staurosporine.

This dual inotropic effect of $PGF_{2\alpha}$ may explain why $PGF_{2\alpha}$ prolonged the contraction-relaxation cycle in the atria, but not in ventricular myocytes (figures 8 & 13). An increase in pH_i under steady-state conditions should prolong the duration of the contraction-relaxation cycle as the affinity of troponin C for Ca^{2+} is increased (Allen & Orchard, 1983). However, the negative inotropic effect of $PGF_{2\alpha}$ seen in ventricular myocytes but not in atria, may oppose part of the action of the alkalosis. The result would be that the time for relaxation appeared unchanged. If this is true, then, the net effect of the $PGF_{2\alpha}$-induced alkalosis in ventricular myocytes would be to increase maximum Ca^{2+} activated force.

4.9 Coronary effects of $PGF_{2\alpha}$

Besides modulating cardiac contractility, $PGF_{2\alpha}$ also modulates coronary tone. $PGF_{2\alpha}$ transiently increased and then decreased coronary perfusion pressure in Langendorff perfused rat hearts (figures 1 & 3).

The transient increase in perfusion pressure implies that $PGF_{2\alpha}$ constricted resistance coronary vessels. The mechanism of this effect was not studied, but it may be mediated partly by sensitisation of the myofilaments to Ca^{2+} (Hori *et al.*, 1993; Suematsu *et al.*, 1991), and also by IP_3-induced sarcoplasmic reticulum Ca^{2+} release (Morimoto *et al.*, 1990a/b; Kurata *et al.*, 1993). This $PGF_{2\alpha}$-induced vasoconstriction may be mediated by TP receptors, as shown in rat aorta (Rapoport, 1993), human hand veins (Arner *et al.*, 1991) and cat coronary arteries (Nakajima *et al.*, 1990).

The prolonged vasodilation induced by $PGF_{2\alpha}$ coincided with the sustained positive inotropic response. This vasodilation may either be metabolically induced, or directly FP receptor mediated. The increased workload and O_2 demand due to the positive inotropic effect could induce the generation of vasodilator metabolites such as adenosine or prostacyclin (Feigl, 1983). In fact, De Deckere and associates (1979) have detected the release of the vasodilator prostaglandins, prostaglandins E_2 and prostacyclin, in the perfusate of isolated rat hearts treated with $PGF_{2\alpha}$. On the other hand, $PGF_{2\alpha}$ may have additional vasodilator actions. $PGF_{2\alpha}$ mediated vasodilation occurs in human hand veins (Arner *et al.*, 1991), and in rabbit jugular veins where nitric oxide is implicated (Chen *et al.*, 1995). Although atrial natriuretic factor is released by $PGF_{2\alpha}$ in perfused rat hearts, it does not appear to contribute to the vasodilation (Rayner *et al.*, 1993).

4.10 Potential regulatory roles of $PGF_{2\alpha}$

The results from this study may explain some of $PGF_{2\alpha}$'s other cardiac effects seen in different experiments.

Cardiac release of $PGF_{2\alpha}$ increases in some pathological situations. Human hearts made temporarily ischaemic by rapid pacing are associated with almost a three-fold increase of $PGF_{2\alpha}$ in the coronary sinus blood (Berger *et al.*, 1977). Rat hearts with infarction and hypertrophy have six-fold more extractable $PGF_{2\alpha}$ than control hearts (Lai *et al.*, 1996). Also, left ventricle samples taken from rabbits that have been subjected to acute pressure overload show up to ten times basal levels of $PGF_{2\alpha}$ (Chazov *et al.*, 1979). The significance of these observations is not clear.

Since $PGF_{2\alpha}$ increases the contractility of ischaemic rat hearts (Karmazyn, 1993), its myofilament Ca^{2+} sensitising action may be beneficial, as opposed to increasing cytosolic Ca^{2+}. Firstly, energy for extruding excess Ca^{2+} through the Ca^{2+} ATPases is not wasted, and secondly, this mode of action would not promote Ca^{2+} overload in the energy deprived, arrhythmia prone, ischaemic heart. However, Karmazyn (1986) reported that $PGF_{2\alpha}$ was detrimental to recovery upon reperfusion of low-flow ischaemic rat hearts. The amount of $PGF_{2\alpha}$ in the coronary effluent also doubled under these conditions. The release of $PGF_{2\alpha}$ may be stimulated by platelet activating factor (Rayner, 1993) and oxygen free radicals (Gupte *et al.*, 1996), which contribute towards development of reperfusion injury. Excessive stimulation of the Na^+/H^+ exchanger, which is already active upon reperfusion of ischaemic hearts, is known to indirectly induce Ca^{2+} overload by perturbation of the Na^+/Ca^{2+} exchanger (Lazdunski *et al.*, 1985, Tani, 1990). Such an action may contribute to the $PGF_{2\alpha}$-induced ventricular failure observed by Karmazyn (1986).

From another point of view, $PGF_{2\alpha}$ may potentially be beneficial in pathological situations because it could mimic ischaemic preconditioning. Preconditioning is a phenomenon whereby a brief period of ischaemia and reperfusion protects the heart against subsequent prolonged ischaemic insult. PKC is believed to be a mediator of this event (Ytrehus *et al.*, 1994; Brooks & Hearse, 1996). Therefore, since $PGF_{2\alpha}$

did activate PKC in my study, it may potentially be protective, as other PKC activators such as bradykinin, angiotensin II and endothelin-1 do mimic ischaemic preconditioning (Liu *et al.*, 1995, Wang *et al.*, 1996).

$PGF_{2\alpha}$ also releases atrial natriuretic factor (ANF) from rat heart and myocytes (Rayner *et al.*, 1993; Lai *et al.*, 1996). This would be beneficial in failing hearts because ANF causes peripheral vasodilation and reduces blood volume, ultimately reducing cardiac afterload. Since $PGF_{2\alpha}$ activates the Na^+/H^+ exchanger, this may play a part in the release of atrial natriuretic factor and the ventricular hypertrophy seen by Adams *et al.* (1996) and Lai *et al.* (1996) in neonatal rat cells. Activation of the Na^+/H^+ exchanger is associated, but not required, to initiate cell growth and proliferation (Noel & Pouyssegur, 1995). Although cardiac hypertrophy enables the heart to adapt to demands for increased cardiac output or injury in the short term, excessive hypertrophy is detrimental.

High concentrations of $PGF_{2\alpha}$ reduce the maximum rate of depolarisation in guinea-pig atria, rat papillary muscle and cultured chick heart cells (Forster *et al.*, 1974; January & Schottelius, 1974; Kecskemeti *et al.*, 1978). This may be due to reduced Na^+ influx through Na^+ channels, as Na^+/H^+ activation reduces the sarcolemmal Na^+ gradient. This indirect Na^+ channel inhibitory effect may explain the antiarrhythmic action of high concentrations of $PGF_{2\alpha}$ (Forster *et al.*, 1973; Vapaatalo *et al.*, 1978).

In summary, the release of $PGF_{2\alpha}$ under pathological conditions appears to be protective in some cases, but is detrimental in others.

4.11 Conclusion

In conclusion, $PGF_{2\alpha}$ appears to activate the Na^+/H^+ exchanger, at least partly by PKC phosphorylation. The resultant alkalosis sensitises the contractile filaments to Ca^{2+}, in the absence of overall changes in Ca^{2+} transients, and this results in the $PGF_{2\alpha}$-induced positive inotropic action in rat ventricular myocytes.

SECTION 5

References and Bibliography

5.1 References and bibliography

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